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ASSESSMENT OF RISKS TO HUMAN REPRODUCTION AND TO DEVELOPMENT OF THE HUMAN CONCEPTUS FROM EXPOSURE TO ENVIRONMENTAL SUBSTANCES

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CHAPTER 1

INTRODUCTION

The U.S. Environmental Protection Agency (EPA) has the legislative mandate to consider regulatory alternatives for chemicals that are causing or can cause a health hazard to man. Because the reproductive system contains some of the more sensitive targets of potentially hazardous agents whose impact on human populations may be immediate, toxicity to the reproductive system and the conceptus is of emerging scientific and social interest. As a result of this interest, the Offices of Health Research and of Health and Environmental Assessment within the Office of Research and Development sponsored a conference to produce a technical document on the current status of risk assessment methodologies for teratogenic and other reproductive effects. The conference brought together scientists knowledgeable in reproductive biology and teratology to discuss techniques and concepts pertinent to developing risk assessment methodologies.

Conference participants were selected based on their expertise in the various disciplines of reproductive biology, statistics, pharmacokinetics, endocrinology, epidemiology, and sexual behavioral toxicology. Draft copies of the report were sent to numerous scientists in academia and the private sector for peer review, and their comments were used by the members of the conference to modify the final document.

The document is divided into three main subject areas: assessment of toxicity to female reproduction, assessment of toxicity to male reproduction, and assessment of toxicity to the conceptus. There are three supplemental parts: pharmacokinetics and epidemiologic considerations, which are common to all toxicological assessments, and a special section on the behavioral aspects of sexual development.

The specific areas addressed in this report are the potential adverse effects on the female and male reproductive systems as well as adverse effects on the developing conceptus. A broad range of problems and effects are discussed, including infertility, early resorption of the conceptus, and possible behavioral disorders produced by subtle changes in the biochemical environment of the fetus.

The report also provides suggestions for improvement in standard toxicological protocols for evaluation of reproductive risks, identifies new concepts and procedures that can be immediately applicable, and designates those that need further expansion and development through research. Included is a discussion on the predictive ability of the tests in estimating risk.

The information in this document will be of value not only to scientists conducting experiments on the effects of chemical agents on the reproductive system, but also to those that need to assess the results from such studies. Thus many tests discussed herein may currently be inappropriate, economically or technically, for regulatory use, but are included to provide necessary and useful background information for evaluating data.

In assessing human risks from exposure to potentially toxic chemicals, many considerations should be addressed, such as severity and reversibility/irreversibility of the effect, existence of threshold or nonthreshold levels, dose-response relationships, sensitivity of the toxicological response evaluated, and predictive ability of animal studies to determine the risk to humans. Attempts have been made in this document to address these considerations.

CHAPTER 2

FEMALE REPRODUCTION

Risk assessment for toxicants that alter reproduction in females involves two separate but equally critical tasks. These are assessment of reproductive parameters in laboratory animals to identify compounds that are prospective reproductive toxicants and continuous epidemiologic surveillance of normal human reproductive characteristics to identify their prevalence, trends, and geographical differences and their potential modification by environmental events. An approach to the evaluation of epidemiologic data is provided in Chapter 5.

The problem of risk assessment has been approached by proposing an animal screening system for qualitative and quantitative analysis of reproductive toxicants. This system is coupled to an integrated data base that serves as a mechanism for the analysis of structure-function relationships of potential toxins. These testing systems form a comprehensive screening scheme that should serve to detect reproductive toxins and provide a foundation for risk assessment. In addition, such a system will serve as a repository of information into which continued input should expand our understanding of risk assessment and reproductive toxicology.

General Reproductive Toxicity Screen

We propose that the stepwise scheme shown in Fig. 1 be followed in an attempt to identify compounds presently in the environment for which there is epidemiologic evidence of adverse reproductive effect and to identify new compounds that may be disseminated into the environment. At the first level, a compound should be tested by the laboratory procedures described below. The standard acute, subacute, and subchronic toxicological testing protocols do not incorporate procedures for detection of reproductive effects, and therefore the following screening procedures were specifically designed for this purpose. If the result of any screening test is positive, the compound must be evaluated by the quantitative risk assessment procedures. If the screening tests are all negative, the compound

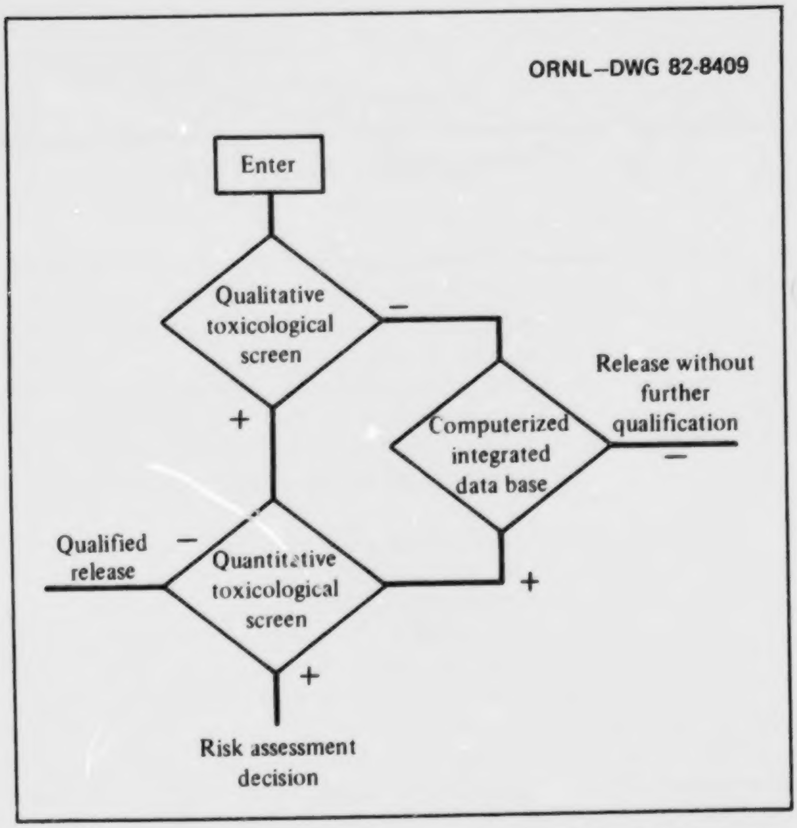
ORNL–DWG 82-8409
Enter
Qualitative toxicological screen
–
+
Computerized integrated data base
Release without further qualification
–
Qualified release
–
Quantitative toxicological screen
+
+
Risk assessment decision

Figure 1 – Reproductive toxicity assessment prospective evaluation.

must then be compared by the computerized integrated data base for structural relationships and/or similarities in the probable pharmacokinetics with other compounds known to affect the reproductive system. An examination of the potential degradation products of the compound, using a computer model analysis of its chemical structure, might also prove useful. If the compound is found to have a structural or functional similarity to known active agents, it must undergo the quantitative risk assessment procedures. If no such affinity is found (and the qualitative screen is entirely negative), the compound can be released into the environment without further testing.

If a compound that enters the quantitative risk assessment procedures is found to be without activity ("false positive" in screens or in search for structural affinity), it can be given qualified approval. That is, its use must be restricted and accompanied by appropriately directed epidemiologic surveillance.

If a compound that enters the quantitative risk assessment procedure is positive, the risk for humans should be estimated, insofar as possible. This information must be weighed and the decision made about whether the compound can be released at all and if so, with what restrictions.

Qualitative Reproductive Toxicity Tests

Estrogen agonist–antagonist

Estrogens mediate, integrate, and modulate interaction of the hypothalamic-hypophyseal-gonadal axis and, as such, are important hormones in the control of reproduction. Exposure to exogenous estrogens is known to have deleterious effects on reproductive potential (1). For predicting the estrogenicity of environmental chemicals, a series of simple screening tests are proposed. These include (a) time of vaginal opening in the neonatal rat, (b) uterine epithelial cellular hypertrophy, and (c) estrogen-receptor-binding analysis. These tests have been chosen because of their sensitivity to estrogenlike substances and the close correlations that exist between these estrogenic responses and subsequent abnormal reproductive capacity. For details of the test procedures, see the Appendix to this chapter.

The tests cited above can be used to detect estrogen toxicants; however, they could also be used to classify estrogen agonist-antagonist. Generally, a decreased response in the tests would indicate an antagonistic effect, whereas an increased response would indicate an agonistic effect. Such a classification scheme, which would require extension and expansion of the tests cited, could form the basis for a structure-function data bank for the prediction of estrogenic toxicity.

Androgen agonist–antagonist

Androgenic substances are known to cause infertility in female animals, and their effects on the human fetus are well known. Exposure to androgens during pregnancy causes masculinization of female fetuses and various physiological and behavioral problems in the adult. Androgenic compounds can be assessed by their capacity to stimulate weight increases in the ventral prostate and seminal vesicles of the intact immature male rat or mouse. This assay is acceptable and practical for predicting androgenic effects (agonistic and/or antagonistic) in humans.

It is important to determine whether a potential toxicant influences development or reproductive capabilities. For this purpose, determining responses of newborn rats following exposure to suitable doses of the potential toxicant provides a multifactorial assay. Similarly, screening tests (e.g., testosterone blood levels or accessory sex gland weight) for androgen antagonists are also available. More sensitive tests for androgen agonists/antagonists are described in the quantitative section of Female Reproduction and in the Appendix to this chapter.

Nonsteroidal toxicant screening tests

The preceding tests will detect estrogenic and androgenic toxicants. For testing of substances other than these two classes of compounds, a multigenerational protocol is proposed. This protocol is designed to evaluate (a) adult female conceptive ability with initial exposure to the agent occurring near puberty, (b) the effect on pregnancy, (c) potential transmission during lactation, and (d) reproductive performance of the second generation. As a standard approach to the testing of potential reproductive toxicants, these tests will detect substances that interfere with reproduction at various levels of biological organization. (For details see the Appendix.)

Computerized integrated data base

The computerized integrated data base should include all known structure-function relationships for reproductive toxicants. With such a data base it would be possible to construct reproductive toxicant profiles (activity profiles) that would predict the potential activity of putative toxicants. Admittedly, such a scheme has shortcomings and prediction will not be perfect. However, if at some future date sufficient information were available in the data base, it could prove to be most useful and time saving.

Quantitative Reproductive Toxicity Tests

The screening tests outlined in the previous section are designed to identify compounds that may represent reproductive hazards.

Once a compound is found to have either a reproductive effect in the qualitative screen or a structural relationship with known active agents in the computerized-integrated-data-base screen, a more detailed quantitative evaluation is mandatory. This process will vary substantially, depending on the type of effects seen in the qualitative screen or the characteristics of the known toxicant to which it appears similar. These tests for quantitative assessment, which are presented in detail in the Appendix to this chapter, can be used to determine site or locus of action of these xenobiotics. It is important to recognize that some xenobiotics may act at more than one site and by more than one mechanism. Ultimately, these specific assays have the potential to determine risk of exposures. In this document an attempt is made to provide an interface between female reproductive biology and toxicology.

Risk Assessment

Assessment of risks to the female reproductive system from environmental sources will have to involve a broad class of potentially affected processes, organs, and structures obtained from human exposure and relevant laboratory results. The reversibility of effects needs to be considered carefully. The applicability of the available information to potential environmental exposure will need to be considered. The magnitude of human risk for reproductive toxicity may be modulated by such diverse factors as distribution of the compound in the environment, patterns of use or exposure, persistence in the biosphere, concentration in the food chain, and age-dependent changes in sensitivity.

Risk assessment will require a knowledge of pertinent factors related to the reproductive process and of relationships of specialized laboratory results to these factors. If a compound demonstrates a reproductive effect in any mammalian species, this observation indicates that some concern about actual human exposure to the agent is justified. Positive results in a number of laboratory tests, which by themselves may be only suggestive of harm, will be important in evaluating potentially detrimental effects.

Substantial modifications in any of the subsystems given in Table 1 are known to be serious and should be avoided. Future testing may indicate relationships between these subsystems and other laboratory testing. Risk assessment for female reproduction requires the establishment of assays relevant to these reproductive processes and the validation of these assays in identifying substances actually toxic to human reproduction. The assays should be shown

TABLE 1 Reproductive Processes Potentially Susceptible to Reproductive Toxicants

	Nonpregnant	Pregnant
Vulva/Vagina	Virilization Adenosis	
Cervix	Structural abnormalities Mucus production and/or quality	Incompetence
Uterus	Luminal fluid Structural malformations Dysfunctional bleeding Dyssynergia Deficient pseudodecidual response	Untimely parturition Dysfunctional labor Uterine blood flow Gestational trophoblastic disease Deficient decidual response
Fallopian Tube	Gamete transport fluid	Zygote transport Ectopic pregnancy
Ovary	Decreased number of oocytes Increased rate of follicular atresia Follicular: steroidogenesis maturation rupture fluid quality Oocyte maturation Luteal function Chronic anovulation	Luteal function
Breast	Supernumerary mammary glands Galactorrhea Nongalactorrheic discharge Gynecomastia	Lactational transport of toxicants Lactation: composition capability
Placenta		Transplacental transport of toxicants Hydatidiform mole Enzymatic activities
Pituitary	Hyperprolactinemia Hypoprolactinemia Altered synthesis and release of trophic hormones	
Hypothalamus	Altered synthesis and release of neurotransmitters, neuromodulators, and neurohormones	
Liver	Metabolism Binding protein synthesis	Metabolism Binding protein synthesis
Adrenal	Steroidogenesis	Steroidogenesis
Behavior	Sexual behavior	Maternal behavior
Reproductive lifespan	Puberty Menopause	

to be relevant in the species used, especially any assays adapted from human assays, and should avoid complications such as those resulting from diurnal variations in hormone level. The results from these studies may indicate other areas where regulation will be necessary.

It is generally felt that when dose-response relationships are observed, lowering exposure will cause less harm when the compound is xenobiotic, unless evidence to the contrary exists. Consideration could be given to using safety factors to establish acceptable exposure levels in situations where harm can reasonably be expected and the exposure cannot reasonably be avoided.

Research Needed

Qualitative reproductive toxicity tests

The scheme for the detection of reproductive toxicity discussed earlier and diagramed in Fig. 1 proposes further investigation in several research areas. One such area is that of structure-function relationships, which are not well understood at the present time. No one would have predicted from the structure of kepone that it would bind to the estrogen receptors and stimulate estrogenic responses. Obviously, much needs to be learned about what constitutes an estrogenic molecule. However, kepone would have been detected as an estrogen by the above tests, and indeed, had more been known about structure-function relationships, it might have been suspected before any tests were performed.

The establishment of reproductive-toxicological profiles and structure-function prediction models has just begun. Much basic information is required before such a system can be realized. Therefore, a strong recommendation is that basic research in reproductive toxicity be supported, with a major emphasis on establishing such models.

An important component of the qualitative reproductive toxicity screen is the computerized integrated data base (see Fig. 1). With such a data base, it should be possible to predict the potential toxicity of putative toxicants. That such a predictive scheme has its faults is well recognized; however, further efforts to realize the potential of such a system should not be discouraged on account of these. In theory, when sufficient information is available concerning structure-function relationships of toxicants, such a prediction scheme may decrease the need for extensive animal testing. For this reason it is recommended that further attempts to establish and validate such a data base be made and also that arrangements be made to update continuously such a facility, with the ultimate intent of perfecting predictive potential.

Quantitative reproductive toxicity tests

The recommendations concerning the qualitative tests also apply to the proposed quantitative tests. The information gained from the quantitative tests extend and interplay with the results obtained from the qualitative tests. Therefore, it is recommended that information gained from the quantitative tests be integrated with that obtained from the qualitative tests to permit an even greater understanding of structure-function relationships.

Specific recommendations

1. The relationship between cellular receptors for toxicants and their mechanism of action should be explored further. Such information can be fed directly into a structure-function data bank such as the computerized integrated data base.

2. More work is obviously needed regarding masculinization of the female, an important problem in reproductive toxicity evaluations. Few data are available on dose responses of these effects, and fewer data exist regarding inhibition (antagonism) of the alteration. Further, extrapolation of these data to humans is not possible, because subhuman primates and humans do not sustain substantial defects of ovulation, whereas sexual behavior is altered. Information presently available is insufficient for determining whether this discrepancy is due to the fetal age at which treatment was administered or to actual differences in sensitivities.

3. *In vitro* model systems are needed (in many areas) for the assessment of reproductive toxicants. For example, model systems for the secretion of gonadotropins by the pituitary cells can be used to study toxicants that influence this process. Currently, almost nothing is known about such model systems, and their value for predicting toxicity is potentially great.

Another important *in vitro* model system in need of development is that of inhibition of steroidogenesis. Although this system has been well characterized for many inhibitors (see the Appendix to this chapter), it has not been exploited for its potential as a test system for toxicants. Continued work and support will be needed to develop these model systems and to relate the information obtained to that gathered from *in vivo* studies.

4. Because little is known about the effects of neuroactive substances such as dopamine and norephinephrine on the hypothalamic-hypophysial complex and about the effects of toxicants on this system, research support should be allocated to this important field, both for the development of new methods and for studies on the mechanism of action.

5. Support is recommended for development of models and research on basic mechanisms in behavioral toxicology, an area in which many unknowns exist regarding reproductive toxicity (see the Appendix).

6. Much work is needed in oocyte toxicity, an obviously important area of concern in which there are incompletely understood age, strain, and species differences in sensitivity of ovotoxicity. For example, preovulatory and growing follicles are most sensitive to toxicity in humans, whereas resting follicles are most sensitive in mice. Similarly, significant differences in sensitivity to oocyte destruction exist between mice and rats. However, evidence from studies exploring the effects of antitumor agents on humans and experimental animals suggests that a compound demonstrated to be ovotoxic in rodents will also be ovotoxic in humans.

Extrapolation of animal data to humans

The primary goal of risk assessment for environmental agents is directed toward adverse effect(s) (injury) in human individuals or human populations. In most cases data are available only in animal model systems; hence it is necessary to extrapolate these findings to anticipated changes in humans. Although extrapolations may be possible, it should be noted that our current understanding of the relationships between hormone exposure and toxic outcomes is not optimal. The following discussion is included to illustrate this point.

An increased rate of vascular disease in women taking oral contraceptive pills has been reported by several investigators (see Kay [2] for review). This has been generally attributed to the estrogenic component of the pill and at first may seem to represent a source of data concerning estrogen levels and toxic effects. However, as Kay (2) points out, the progesterone content of the pill, not the estrogen content, is correlated with increased incidence of vascular disease. Progesterone has also been shown to decrease high-density lipoprotein (HDL) cholesterol, an event associated with increased risk of arteriosclerosis. Estrogens increase HDL cholesterol and therefore would be expected to decrease the incidence of vascular disease. Obviously, predicting risks based on estrogen levels in women taking the pill requires further consideration.

This example points out the need for more research at all levels, from biochemical to epidemiological, and emphasizes the requirement for more data before meaningful extrapolations can be made for risk assessment in humans.

REFERENCES

1. McLachlan, J.: Estrogens in the environment. Elsevier/North Holland: New York; 419 pp., 1980.
2. Kay, C. R.: The happiness pill? J. R. Coll. Gen. Pract. 30: 8–19, 1980.

APPENDIX

DETAILS OF TEST PROTOCOLS AND GLOSSARY OF TERMS FOR FEMALE RISK ASSESSMENTS

I. DESCRIPTION AND DISCUSSION OF TESTS USEFUL IN ASSESSING RISK TO THE FEMALE REPRODUCTIVE SYSTEM

Qualitative Reproductive Toxicity Tests

Estrogen agonist–antagonist

Time of vaginal opening in the neonatal rat. Rats are injected on days 1, 3, and 5 of postnatal life, and the time of vaginal opening is noted. Estrogen agonists such as diethylstilbestrol (DES), clomiphene, and tamoxifen are known to cause early maturation of vaginal development (1–3), and this test serves as a sensitive indication of such activity. The general protocol for this test for estrogen agonist is shown in Table 2.

Uterine epithelial cellular hypertrophy. Neonatal rats are treated as described in Table 2, and the uteri are taken on day 7 for histological examination. Epithelial cell growth is an excellent indicator of estrogenicity and will detect compounds, such as clomiphene, which exhibit differential cell stimulation (4). Kepone, DES, dichlorodiphenyltrichloroethane (DDT), and zearlenone have been shown to be either active in this test or very likely active because of their known ability to stimulate uterine growth (4, 5).

The above tests, requiring a minimal number of animals, are simple and reliable. These tests are used routinely in many laboratories and are quite sensitive to estrogenic compounds (μg/kg).

Estrogen-receptor-binding analysis. Uteri obtained from 7-day-old rats which have been treated as described above are examined for nuclear binding of the estrogen receptor by the nuclear exchange assay (4). In the same tissues the quantity of cytoplasmic estrogen receptor can also be determined. This test gives a measure of the

TABLE 2 Estrogen Agonist Screen

Age (days)	Treatment of female rats
0	Birth
4	Beginning of daily dosing for 4 days
7	Sacrifice
	Assays:
	1 – uterine weight
	2 – endometrial histology
	3 – estrogen receptor assays
	4 – vaginal opening[a]

[a] Vaginal opening may occur by day 7; however, a longer time interval after birth may be required (up to 20 days).

ability of a toxicant to bind to estrogen receptor *in vivo* and to cause nuclear accumulation of the receptor-ligand complex. Classical estrogens such as estradiol and DES are known to perform this function, which is presumed to be an obligatory step in the mechanism of action of estrogens. Kepone, DDT, and zearlenone bind to the estrogen receptors, cause nuclear accumulation, and stimulate uterine growth (3, 5 6). Therefore, these compounds are likely to be active in the other tests for estrogenicity and will make excellent reference compounds for testing the model.

Androgen agonist–antagonist

These tests are standard and require no further explanation.

Nonsteroidal toxicant screening tests

This protocol is designed to evaluate: (a) ability of the adult female to conceive with the initial exposure to the agent near puberty (P-generation, Table 3); (b) the effect on pregnancy (live birth index of F_1, F_1^1, F_2, F_2^1 generations); (c) potential transmission during lactation (survival index of F_1 and F_2 generations); and (d) reproductive performance of the second generation (live birth index of F_1 and F_2 generations). Part of the P_1 generation (P_1^1) is mated again at the time of postpartum estrus, because at that time mating behavior, ovulation, implantation, and fetal resorption are more sensitive to environmental disruption than they are during mating at a cycling estrus.

TABLE 3 Time Table for Intergenerational Protocol to Evaluate Putative Toxicant Effects on Reproduction in Sexually Mature Animals

Approximate Time (weeks)	Parent (P_1) Generation (female)	F_1 Offspring	F_2 Offspring
0	P_1 – born		
6	P_1 – dosing begins		
10	P_1 – mated		
~13	P_1 – bears and suckles F_1 litter (dosing continued)		
	P_1'[a] – postpartum mated (dosing continued)		
~16	P_1' – bears F_1' litter[b]	F_1 – weaned, dosing begins	
~23		F_1 – mated first time (females)	
~26		F_1 – bears first litter (F_2)	
~29			F_2 – weaned, sacrificed
~40		F_1 – mated second time	
~43		F_1 – bears second litter (F_2')[c]	

[a] Represents that portion of the P_1 generation that is mated at postpartum estrus.

[b] The F_1' litter is used as a check for the reproductive efficiency of the P_1' postpartum mating.

[c] The F_2' litter is used as a check for the reproductive efficiency of the second F_1 mating.

The age at which the animals are mated is a major procedural factor that can influence the fertility test results in the F_1 and F_2 generations. Toxicants, particularly those that possess steroidal activity, will diminish the success of pregnancy and number of offspring of older females but not those of younger females. Thus, while testing at earlier ages is more economical, it might yield false negative results. Therefore, a portion of the F_1 generation should be examined for ovarian cyclicity and fertility at approximately six months of age (live birth index of F_2^1 generation).

Experimental, vehicle control, and positive control (use of a known toxicant to verify the system) groups should be utilized with at least 20 animals per group. Selecting the agent to be used as a positive control will be arbitrary, and species or strain differences may complicate the choice. Despite these drawbacks, inclusion of a positive control that most appropriately parallels the test compound would seem mandatory for validation of the test system. The entire protocol need not be completed if adverse effects are demonstrated early in the protocol (i.e., live birth index of the F_1 generation).

The maximum tolerated dose should be used. Other dose levels may be included if dose response information is needed. Route of administration should be in food or water to avoid handling pregnant and lactating females, which may result in stress independent of that potentially caused by the agent being tested. This may complicate quantification of the ingested dose but ensures continuous dosing of the F_1 generation during weaning. Other routes of exposure (e.g., gavage or parenteral administration) may be used if the test protocol can be modified to avoid any interfering stress. Dosing begins at six weeks of age of the P_1 generation and continues until the end of the protocol. Body weights should be recorded weekly for all animals in the P_1 and F_1 generations as well as pup weights in the F_1, F_1^1, F_2, and F_2^1 offspring.

P_1 females are mated with untreated males of proven fertility at ten weeks of age in a one-to-one sex ratio. Successful mating is determined by finding a copulation plug and presence of sperm in the vaginal smear. These same females are then mated again at the time of postpartum estrus, 8–10 hours after giving birth. Twenty females from the F_1 generation (the offspring resulting from the first mating), are randomly selected and mated with untreated males of proven fertility at ten weeks of age. The offspring of postpartum mating (F_1^1) need only be counted and weighed at birth.

The selection of the species of the test animal to be used in the toxicant screening procedures will be determined by several considerations including cost, time, and ability to assess related human reproductive processes. The rhesus monkey or other subhuman primates, being a more comparable reproductive species, would be the animals of choice, but their cost as well as other considerations would prohibit their use in screening procedures. Laboratory rodents are economically feasible, but the relevance of the outcome of the screening to the human could be questioned. On the basis of current information, different species and strains will have to be selected for evaluating different components of the human reproductive system. Each choice would carry with it a risk of obtaining false positive and false negative data with regard to the relevance to the human female. For example, on the basis of contemporary results, the rat would be less satisfactory than the guinea pig for assessing the effects of potential toxicants on the development or the integrity of cyclic gonadotropic function.

Several neural and physiological interventions that curtail estrous cycling in the rat do not occur in the rhesus monkey and guinea pig. In addition at least some perinatal steroid manipulations that render the rat permanently anovulatory apparently do not interfere with menstrual cycles in the rhesus monkey. Thus, it is likely that many substances found to disrupt spontaneous ovulation in the rat will not do so in the human, and false positive assessments may result.

A false negative may occur if the rat is the only species used to assess the reproductive consequences of a compound. For example, the ovarian cycle of the rat does not have a spontaneous luteal phase as does the human cycle. Therefore, compounds that might interfere with the function of the corpora lutea cannot be detected in the rat. Under these circumstances another species with a comparable reproductive process, such as the guinea pig, should be considered for addition to the screening procedure.

Indexes should be calculated for mating, fecundity, female fertility, and parturition as noted below.

$$\text{mating index} = \frac{\text{number of copulations (one counted/estrous cycle)}}{\text{number of estrous cycles required}} \times 100$$

$$\text{fecundity index} = \frac{\text{number of pregnancies}}{\text{number of copulations}} \times 100$$

$$\text{fertility index} = \frac{\text{number of females conceiving}}{\text{number of females exposed}} \times 100$$

$$\text{incidence of parturition} = \frac{\text{number of parturitions}}{\text{number of pregnancies}} \times 100$$

Numbers of viable, stillborn, and cannibalized progeny are recorded for each litter, the survivors on days 1, 4, 12, and 21 postpartum noted, and litters reduced to ten pups on the fourth day of lactation for standardization. Gestational length and sex ratio are also monitored.

$$\text{live birth index} = \frac{\text{number of viable pups born}}{\text{total number of pups born}} \times 100$$

$$\begin{array}{l}\text{1- or 4-day survival}\\ \quad\text{index}\end{array} = \frac{\text{number of pups viable at location day 1 or 4}}{\text{number of pups born}}$$

$$\begin{array}{l}\text{12- or 21-day survival}\\ \quad\text{index}\end{array} = \frac{\text{number of pups viable at lactation day 12 or 21}}{\text{number of pups retained at lactation day 4}}$$

Quantitative Reproductive Toxicity Tests

Estrogen agonist–antagonist

The screening test listed previously under qualitative assessment can be used to establish dose-response relationships between estrogens and suspected estrogenic toxicants. The following discussion represents an expansion of the qualitative tests.

Neonatal exposure to various dose levels of estrogenic toxicants. These assays will result in a dose-response relationship for time of vaginal opening, ovarian degeneration and oocyte loss, and stimulation of epithelial cell height in the uterus. These end points are easy to assess, are reproducible, and are quite sensitive (μg quantities of DES, Kepone, and clomiphene are easily detected) (1–3). This is not to say that these tests have been utilized to examine a large class of compounds; however, one of the recommendations is that such compounds be studied in detail. At the present time all known estrogens are active in these assays, and hence we can expect that they will be good predictors of estrogenic potency. Likewise, such assays should identify compounds that may interfere with reproductive processes. It may be possible to extrapolate these data on relative potency to known effects of various doses of estrogens in the human, since it is well established that estrogenic responses in rodents and humans show many similarities (7–9). To this end compounds such as ethynylestradiol, DES, and estradiol should be used as standards.

Estrogen receptor analysis *in vivo* and *in vitro*. An extremely sensitive (picogram-nanogram range) and reproducible method for assessing relative estrogenicity involves the use of toxicants to compete with labeled estradiol in binding to uterine cytoplasmic estrogen receptors (3–6). This test involves the addition of various concentrations of the toxicant to uterine cytosol fractions in the presence of labeled estradiol. If the toxicant is estrogenic, it will compete with estradiol for binding to receptor sites, and a classical competitive inhibition curve can be obtained. From this curve a relative binding affinity (RBA) can be calculated that reflects the agonistic or antagonistic activity of the toxicant. Such estimates of potential estrogenicity may be used to extrapolate estrogenicity in humans and be of importance in approximating the relative risks in humans. Although this test is simple and requires little expense in terms of number of animals, etc., not all laboratories routinely perform such analyses. However, it is becoming more and more common and may be standard procedure in the future.

The major qualifier to such cytosol receptor assays is that certain estrogenic compounds, such as clomiphene and nafoxidine, exhibit a very low RBA and yet are more estrogenic than predicted (10). In part this is due to the slow clearance of such compounds, which provides a longer exposure time and increases the receptor occupancy *in vivo* when compared to that of more rapidly cleared estrogens. To detect such long-acting estrogens, estrogen receptors assays can be done *in vivo*. Mentioned in the section titled Qualitative Reproductive Toxicity Screen, these assays involve injecting various dose levels of the compound in immature rats and measuring the nuclear accumulation and cytoplasmic depletion of estrogen receptors. Reliable, easy to perform, and sensitive, this test requires relatively few animals. It has the disadvantage of not being a standard assay in all laboratories.

Such receptor assays can be valuable in the estimation of estrogenic potency in humans; however, the chief value of the receptor assay probably lies in its ability to detect estrogen agonist or antagonist and has the potential of elucidating primary steps in the mechanism of action of such compounds. Such insights into mechanisms may make future predictions of estrogenic toxicity a relatively simple task.

Androgen agonist–antagonist

Qualitative screening tests for androgen activity include the ventral prostate gland hypertrophy produced by administration of compounds to immature (28-day) male rats.

Additional models are necessary to evaluate androgenic effects in the following circumstances: (a) inhibition of adult female reproductive function (e.g., ovulation, behavior), and (b) masculinization of female phenotype (fetal differentiation, prepubertal development, and adulthood phenotypic transformations).

Inhibition of adult female reproductive function. The common clinical response to hyperandrogenic stimuli, is anovulation. Increasing duration of exposure or potency of the agent leads to oligomenorrhea and secondary amenorrhea. Subtle intensifications of libido are experienced by some women, particularly with more potent agents.

Laboratory testing of adult female rats requires daily evaluation of vaginal smears for no fewer than four cycles to detect interruption of the estrous cycle. A daily 1-mg dose of testosterone propionate produces diestrus within two cycles. Appropriate dose-response studies are indicated.

Masculinization of the female phenotype: fetal. After 16 days of gestation, transplacental transfer of potent androgen agonists results in a variety of imprinting and masculinizing responses that are based upon "critical periods" of organ system differentiation. Permanent alterations in the neuroendocrine regulation of the estrous cycle and male-type mating behavior are "imprinted" at lower doses of androgen than are required for disturbing reproductive tract (vaginal opening) and hepatic monooxygenase (steroid hydroxylase or dehydrogenase) activities. A 5-mg dose of testosterone propionate administered to the pregnant dam daily from day 16 to day 20 of gestation produces the masculinization response in female progeny and does not significantly disturb male differentiation. Treatment of neonatal female rats (day 1–10) with a single 1-mg dose of testosterone propionate masculinizes the hypothalamic-pituitary-ovarian axis (persistent estrus) and sexual behavior (male-type with great reliability).

Masculinization of the female phenotype: postnatal animals. Masculinization of the female phenotype and suppression of female sexual behavior and of the pubertal events is not induced permanently by treatments initiated after the postnatal period (days 1–10). Such masculinization effects produced in females tend to regress, and although vulvar changes may persist, estrous cyclicity resumes. Although these effects are clear-cut in rodents, dose extrapolation to humans is not possible.

Adulthood phenotypic transformations. Masculinization of vulva, mating behavior, and hepatic monooxygenases in adult animals are much less sensitive indicators of toxicity than the same responses in immature animals. Inhibition of ovulation in adult females is also a more sensitive indicator of toxicity than the above three parameters. Therefore, additional tests to assess phenotypic transformation in adult females are not necessary.

Hypothalamic–Pituitary Function Tests

Assay of agents that stimulate the release of gonadotropins from cells of the anterior pituitary gland

A toxicant may adversely affect reproduction by altering the rate of secretion of one or more hormones that are synthesized and released by the hypothalamus and anterior pituitary gland. Of the hormones that are secreted by the anterior pituitary gland, the gonadotropins (luteinizing hormone [LH] and follicle-stimulating hormone [FSH]) and prolactin are most closely associated with reproduction. The gonadotropins are important, because these protein hormones control ovarian function, including steroid hormone secretion, follicular development, and ovulation. Hence, if gonadotropin secretion is suppressed, ovarian function is suppressed. A toxicant could suppress the secretion of gonadotropins by acting directly on the pituitary gland or by suppressing the secretion of gonadotropin-releasing hormone (GnRH) by hypothalamic neurons.

Alternatively, a toxicant could stimulate the secretion of prolactin, and as a consequence of the hyperprolactinemia, gonadotropin secretion becomes suppressed. Prolactin secretion can be stimulated by substances that have estrogenic activity, substances that act as dopamine antagonists, substances that inhibit dopamine secretion by hypothalamic dopaminergic neurons, or substances that cause hyperplasia of prolactin-secreting cells. Some of these actions of toxicants can be assessed (e.g., by quantifying gonadotropin and prolactin secretion), whereas others cannot be evaluated in a quantitative sense (e.g., GnRH secretion). A few ways of assessing quantitatively the actions of a toxicant that may have significant effects on reproduction are listed below.

***In vivo* model.** Since agents that stimulate the release of one gonadotropin (e.g., LH) usually affect the release of the other (viz., FSH), it is probably only necessary to measure the release of one (e.g., LH). For such studies, the estrogen-progesterone-primed female rat can be used. The Gn-releasing standard should be synthetic GnRH against which the test substance can be compared. The responsive parameter, LH in serum or plasma, can be measured by a standardized radioimmunoassay. After the ED_{50} of GnRH and the ED_{50} of the test substance have been ascertained, a standard bioassay can be performed. A three-dose assay, where each dose is replicated three to four times, may suffice. Thus, such an assay will require 18 to 24 assay animals. More animals can be used if high precision is desired. The concentration of LH in plasma or serum can be evaluated 30 to 60 minutes after the administration of the test substance and of the standard. Assuming suitable ranges of concentration and parallel slopes from the two assays and a suitable statistical analysis, such as that described by Bliss (11) for bioassay qualitative characteristics of the assay as well as the relative potency of the unknown substance can be evaluated. If one knows the potency of GnRH in the estrogenized woman with reference to the release of LH, it is then possible to calculate the Gn-releasing activity of the test substance and express the potency in terms of GnRH.

***In vitro* model.** The Gn-releasing properties of an unknown substance can also be measured using anterior pituitary cells maintained in monolayer culture. In this case the pituitary cells could be obtained from the rat or a suitable primate. The cells could be dispersed and established in culture. After three to five days, the test substance and GnRH can be assayed for their Gn-releasing activities, using a bioassay paradigm similar to that outlined above.

Assays of agents that inhibit the release of gonadotropins from cells of the anterior pituitary gland

The details of an *in vivo* model assay of a substance that inhibits the release of gonadotropins could be done as follows. A female rat castrated 4 to 6 weeks before testing could serve as the assay animal. (In such an animal, the concentration of LH is many times that of intact animals.) For a reference standard, 17β-estradiol could be used to suppress the release and hence concentration of LH in serum of the test animal. After the ED_{50} for estradiol and the ED_{50} for the test substance have been established, a three-dose bioassay could [illegible] conducted. After an evaluation of the parameters of the assay, it may be possible to calculate the relative potency of the test substance and express its potency in terms of 17β-estradiol.

After the potency of a test substance relative to 17β-estradiol has been established, one can then calculate the relative potency of the test substance in the human by comparing the LH-lowering effect of 17β-estradiol in castrated or postmenopausal women.

Assay of an agent that inhibits the release of prolactin from cells of the anterior pituitary gland

***In vivo* model.** Although several experimental animal models could be used, the young, mature female rat would be adequate. The only pretreatment required would be a short period (3–4 days) during which the animal was handled to minimize the release of prolactin because of fright. The reference standard for the release of prolactin could be haloperidol. Prolactin release could be evaluated by measuring prolactin in the plasma or serum of the test animal. Thirty to sixty minutes after the administration of the test substance or haloperidol, serum or plasma prolactin could be measured by radioimmunoassay. After the dose-response curve (i.e., ED_{50}) is established, a bioassay could be conducted and the relative potency of the test substance calculated.

If a dose-response curve for haloperidol in women is established, one could approximate the potency of the test substance relative to haloperidol. Of course, other reference standards could be used in this prolactin release assay *in vivo*. These include thyrotropin-releasing factor and vasoactive intestinal peptide.

***In vitro* model.** The ability of test substance to simulate the release of prolactin from pituitary cells could be conducted *in vitro* using pituitary cells maintained in monolayer culture. The donor could be the rat as well as a primate. After a few (3–5) days in culture, a suitable bioassay could be performed, and the potency of the test substance relative to a standard could be evaluated.

Assay of an agent that inhibits the release of prolactin from pituitary cells

***In vivo* model.** An estrogen-primed female rat could be used in this assay. In such an animal the plasma concentration of prolactin is very high. For a reference standard, bromoergocriptine could be used. After dose-response curves for bromoergocriptine and for the test substance had been established, a bioassay for prolactin release inhibition could be performed, where the serum or plasma prolactin concentration is the responsive variable. After a suitable statistical analysis, one could calculate the relative potency of the test substance. As discussed above, if the dose-response relationship for bromoergocriptine in the woman (perhaps an estrogenized woman) were known, the approximate potency of the test substance relative to bromoergocriptine could be calculated.

***In vitro* model.** Anterior pituitary tissue from estrogenized female rats can be used under *in vitro* conditions to test a substance for inhibition of prolactin release. Anterior pituitary tissue can be incubated in the presence of various concentrations of bromoergocriptine or of the test substance to establish a dose-response curve. Then, using this system, a bioassay can be conducted where the concentration of prolactin in the culture medium is the responsive variable. Pituitary cells maintained in monolayer culture could also serve as a suitable *in vitro* assay system.

Assay of the activity of an agent that alters the secretion of dopamine by hypothalamic neurons

There is no method for the quantification of the secretion by dopaminergic neurons in the human. Although there is a method for the measurement of the secretion of dopamine into hypophysial portal blood, the procedure is tedious and requires the aid of highly skilled people. Hence, this procedure is not practical as a routine procedure. Therefore, one is reduced to making turnover measurements, but such measurements are also susceptible to large error and require many animals. Thus, it is reasonable to conclude that as a routine matter, the rate of secretion of dopamine by neurons of the brain can not be done for toxicants. This is not to infer that this is not an important aspect of brain function. Indeed, it is already known that the secretory activity by dopaminergic neurons is quickly, markedly, and sometimes permanently affected by a variety of toxicants. Since dopaminergic neurons constitute an important subset of the neurons of the brain, we encourage research on this important topic.

Assay of the activity of an agent that alters the secretion of norepinephrine by hypothalamic neurons

Comments made about the secretion of dopaminergic neurons are equally applicable to neurons that secrete such biogenic agents as norepinephrine and serotonin. The available techniques for th quantitative study of neurons secreting these agents are n sufficiently advanced to enable their use in routine assays.

Assay of the activity of an agent that alters the secretion of GnRH

There is no suitable assay for such an agent at this time.

Assay of the activity of an agent that alters the secretion of hypothalamic opioid peptides

It is now clear that morphine and opiatelike peptides affect the secretion of dopamine by hypothalamic neurons and of LH by the pituitary gland. Thus, it is easy to infer an important role for the naturally occurring opiatelike peptides as well as morphine in reproduction. However, this field is too new to address in a quantitative manner or to include in a screening system. Yet it can be anticipated that at some time in the reasonable future, this shortcoming in our technical capabilities will be surmounted and these problems addressed in quantitative terms.

Blood flow of the hypothalamic-hypophysial system

Perhaps no structure in the mammal has a more complicated vasculature than the hypothalamic-hypophysial complex, consisting as it does of one component that has a high rate of perfusion and another that is avascular. Moreover, the coexistence in the pituitary stalk of portal vessels carrying blood to the anterior lobe of the pituitary from the hypothalamus and of a subependymal plexus in which pituitary hormones can pass retrograde in the stalk to the hypothalamus attests to the importance of blood flow in this area. The measurement of blood flow to the neurohypophysis (i.e., medium eminence and pars nervosa) can be measured accurately using radiolabeled microspheres. Blood flow in the anterior lobe of the pituitary can be measured using the hydrogen electrode. Thus, an area deserving of attention for effects of environmental toxicants is the hypothalamic-hypophysial complex.

Sexual behavior tests

Introduction. Alterations of mating behavior in the female rat can be used as an indicator of hypothalamic function and/or impairment of function. A voluminous literature indicates that hypothalamic neurons serve as target cells for the ovarian hormones, especially the estrogens, and that destruction of specific regions of the hypothalamus leads to abolition or disorganization of female sexual behavior (12). The studies carried out on the disturbance of sexual behavior associated with hypothalamic damage establish three points of significance to the use of the proposed sexual behavior tests: hypothalamic damage can disrupt sexual behavior without altering the neural systems that mediate pituitary-ovarian function, the disruption of sexual behavior following hypothalamic damage cannot be reversed with endocrine therapy, and the sexual disruptions show extensive phylogenetic continuity.

For these reasons, the sexual behavior system may be capable of detecting chemically induced abnormalities in hypothalamic function that cannot be detected by the other testing systems proposed. By substituting the putative toxicant for estrogen or progesterone in a standardized behavioral assay, it is possible to assess its estrogenic or progestogenic activity in hypothalamic regions other than those that modulate pituitary release of gonadotropins. Neural systems modulating female sexual behavior are by no means limited to hypothalamic structures. The tests proposed here, however, are oriented toward behavioral end points generally accepted as involving the hypothalamus. A more detailed discussion of other aspects of sexual function and the potential toxicant-induced disruption of other neural systems is available in Chapter 5.

Behavioral assay methods. Female rat sexual behavior has several components that vary in a dosage-dependent manner with estrogens and progestins. To determine whether the toxicant possesses estrogenic or progestagenic action, the experimenter would vary the dosage of the toxicant, substituting it for either estradiol benzoate (EB) or progesterone in the standard protocol of a behavior assay. The lordosis response, which includes arching of the back (13), and the number or latency of approaches that the female makes to the male (14) can be readily quantified. The testing arena should have an area of at least four square feet and contain a simple barrier or compartment. The females used in the tests should be ovariectomized and administered estrogen and progesterone (or the substituted toxicant) at times to produce mating during the dark phase of illumination. The estrogen EB (or its substitute) is administered 44 to 46 hours before the administration of progesterone (or its substitute), and the mating behavior is observed approximately four hours after administering progesterone.

A standard dose-response curve for EB would be obtained by holding the amount of progesterone constant at approximately 0.1 mg and varying the dosage of EB from 0.1 to 100 μg. When possible, the vehicle for delivering these hormones should be the same as that to be used for the toxicant and appropriate standard curves established. The progesterone standard dosage-response curve would be obtained by maintaining EB at a constant level (5–10 μg) and varying the amount of progesterone from 0.1 to 10 mg. No fewer than 10 subjects can be used to establish the behavioral response value at each dosage. Repeated mating of the same female at each dosage may be more sensitive than using different females at each dosage, but each mating should be separated by approximately 5 to 7 days.

A reliable method to determine the dose-response curve for antiestrogenic activity would be to use guinea pigs as subjects and to measure their lordosis response to the touch of the experimenter; running the index finger along the back, starting from its most caudal point. The procedure would consist of administering constant dosages of EB (5–10 μg) and, 46 hours after the initial dose of EB, a 0.5-mg dose of progesterone. When administered simultaneously with estrogen, progesterone possesses antiestrogenic properties in the guinea pig and can therefore be used as a standard to assess the antiestrogenic activity of the toxicant. Accordingly, varying dosages of the compound (0.1–10 mg) would be administered along with the EB.

The antiestrogenicity of a putative toxicant can also be evaluated in the female rat's behavior system. However, because this behavior system is relatively insensitive to the inhibitory actions or antiestrogenic actions of progesterone, an alternative antiestrogen, such as MER-25, is recommended for a comparative standard.

The toxicant can also be administered in addition to the standard doses of EB or progesterone. This can be done at the same time that estrogen and progesterone are administered to determine whether it potentiates or antagonizes the action of each of these steroids. In addition, the toxicant can be administered prior to the determination of a standard dose-response curve, and the estrogen dose-response curves can be compared with those obtained from control animals. In this way, toxicant-produced modifications of the brain areas mediating sexual behavior can be detected using a test based on a standard estrogen dose-response curve.

Relevance to humans. A positive result from these behavioral screening procedures could reflect disruption of neural, most likely hypothalamic, function and could indicate the potential for interference with human hypothalamic function. However, the manifestations of this potential hypothalamic disruption in humans will most likely be different from those in rodents.

Ovarian Toxicity

Oocyte and follicle toxicity

The ovary is responsible for two roles in reproduction: nurture and release of gametes and hormone production. Clinical and experimental data demonstrate that a variety of xenobiotic compounds can alter both aspects of ovarian function (15, 16). Multiple studies have demonstrated that one of cigarette smoking's adverse effects on the human ovary is an earlier dose-related age of menopause. The assays described here are designed to assess the effect of xenobiotics on the first aspect of ovarian function, gamete nurture. Tests for xenobiotic effects on oogenesis can be determined by including prenatal as well as postnatal treatments. Xenobiotic destruction of oocytes is of great significance because the effect is irreversible: there is no mechanism for repopulation of oocytes in th ovary.

Evidence suggests that inbred mouse strains represent the most sensitive test strains for oocyte and follicle toxicity assays (17). Additional data in other species and with other xenobiotics is needed to clarify this relationship. Inbred mouse strains also offer the advantage that they provide the logical framework for exploration of the mechanism of action as well as providing a reproducible assay system (18–20).

After treatment with the compound of interest, mice are sacrificed at varying time intervals and their ovaries removed, fixed, serially sectioned, and stained. Oocytes and follicles are quantitated using a microscope, and effect of treatment on oocyte or follicle number is determined. Follicles and oocytes are classified by the method of Zuckerman (21). This assay, although cumbersome, is easily learned and conducted by laboratory technicians.

Evidence suggests that this assay is a much more sensitive indicator of oocyte or follicle damage than alterations in fertility. Unpublished investigations at the Pregnancy Research Branch of the National Institute of Child Health and Human Development, as well as other published data, suggest that as many as 90% of all oocytes have to be destroyed before short-term alterations in fertility of the female can be observed.

The full range of specificity of the assay has yet to be determined. The assay appears to be quite sensitive and do dependent (see Table 4 for available ED_{50}'s).

Inhibition of steroidogenesis

In developing a model system to estimate the quantitative risk of a toxicant with regard to inhibition of ovarian steroidogenesis, multiple physiological and technical aspects must be considered. These include (a) the cell-type specific sex steroids to be measured,

TABLE 4 Compounds Tested for Oocyte/Follicle Toxicity in the Murine Assay

Compound	Response	Follicle Type	ED_{50} (mg/kg)
Benzo(*a*)pyrene	Toxic	1-3a[a]	10
3-Hydroxybenzo(*a*)pyrene	Toxic	1-3a	>100
4,5-Dihydroepoxybenzo(*a*)pyrene	Toxic	1-3a	>100
cis-4,5-Dihydrodiolbenzo(*a*)pyrene	Toxic	1-3a	>100
trans-4,5-Dihydrodiolbenzo(*a*)pyrene	Toxic	1-3a	>100
7,8-Dihydrodiolbenzo(*a*)pyrene	Toxic	1-3a	<1
7,12-Dimethylbenz(*a*)anthracene	Toxic	1-3a	<1
3-Methylcholanthracene	Toxic	1-3a	10
Ethanol	Nontoxic		
Benzene	Nontoxic		
Toluene	Nontoxic		
Carbontetrachloride	Nontoxic		
Galactose	Toxic with prenatal exposure, nontoxic after birth	1-3a	Unknown

[a] Resting follicles as classified by Pedersen and Peters (22).

(b) the cooperative compartmental steroid biosynthesis characterizing the follicular phase, (c) the cycle-related variations in sex steroid production, (d) the key regulatory steps in steroid biosynthesis (i.e., the availability of substrate and the roles of luteinizing hormone [LH] and follicle-stimulating hormone [FSH]), (e) the available methodology, and (f) the ability to extrapolate the data between species. The focus here is placed on a model *in vitro* rather than *in vivo* because of considerations regarding the specificity of the toxic effect, the lack of interference by nervous or humoral factors present *in vivo*, the greater likelihood of intracellular interaction with the toxicant, the applicability of the test system, and the ability to extrapolate the data to human or at least primate ovarian cell types.

Estrogen, primarily 17β-estradiol progesterone, 17α-OH progesterone, androstenedione, and testosterone, are the predominant steroids produced by the human ovary during the reproductive years. Estrogen characterizes the follicular phase, with the corpus luteum producing both estrogen and progesterone and a drop in both steroids occurring at the time of menses in a nonconceptive cycle. Androgens are secreted throughout a nonconceptive cycle, with a slight rise at midcycle. Controversy still exists concerning the cell(s) of origin of follicular estrogen; both direct thecal cell secretion and granulosa cell aromatization of thecal androgen are supported in the literature (23). Because granulosa cells lack the 17,20 desmolase enzyme, the thecal and interstitial compartments are felt to be the source of C_{19} androgens. After ovulation the granulosa and thecal compartments both form the corpus luteum and produce progesterone and estrogen. Any model system using ovarian cell types *in vitro* must consider these differences as well as the overall cyclic steroid secretory pattern characteristic to the species utilized.

Regulatory steps in gonadal steroid secretion include (a) substrate (cholesterol) availability (i.e., the low-density lipoprotein fraction of plasma); (b) luteinizing-hormone (LH) induction of the 20,22-hydroxylase-desmolase steps converting cholesterol to pregnenolone, and (c) follicle-stimulating-hormone (FSH) induction of granulosa cell aromatase activity converting thecal androgens to estrogens. Since thecal steroidogenesis has not been demonstrated to depend on FSH-induced aromatization, LH stimulates thecal androgen production, and low levels of LH are required *in vivo* for adequate luteal function, some of these regulatory steps may be compartment specific.

A toxicant may not demonstrate inhibition of steroidogenesis *in vitro* and yet be active *in vivo*, if it affects selectively gonadotropin-mediated events *in vivo* or only progesterone synthesis stimulated by human chorionic gonadotropin (HCG) (24, 25), and hence be detectable only *in vivo* in a known conceptive cycle. Similarly, agents acting through prostaglandins known to induce luteal regression *in vivo* in some species (26) may be active only *in vivo*, because the agents may act not directly on the steroid-secreting cell but rather indirectly by selective ovarian veno-constrictive action. Despite these possibilities, most known inhibitors of steroidogenesis act by affecting specific enzymes in the steroid pathways (Table 5).

TABLE 5 Agents That Inhibit Steroidogenesis

Steroidogenic Step	Inhibitor
20α hydroxylase	Amino-glutethimide phosphate
Sidechain cleavage	3-methoxybenzidine
Dehydrogenase, 3β-hydroxy-Δ^5-steroid	Cyanoketone Estrogens Azastene Danazol
Aromatase	4-acetoxy-androstene-3,17-dione 4-hydroxy-androstene-3,17-dione 1,4,6-androstatriene-3,17-dione
11β-hydroxylase	Danazol Metyrapone SKF-12185
21-hydroxylase	Danazol
17α-hydroxylase	Danazol SU-9055 SU-8000
17,20 lyase	Danazol

Adequate methodology is currently available for (a) isolation of ovarian cell types (27, 28), (b) tissue or organ culture, and (c) direct radioimmunoassay of media for individual steroids without chromatography steps. If HCG stimulation of steroidogenesis is required to demonstrate an effect, serum-free media may be required, as there is some evidence that blocking factors for gonadotropins are present in serum (29); but in short-term cultures (<24 hours), the lack of serum factors should not present a problem for cell viability. Plating efficiency can be determined by supravital staining, and cell counts or determinations of DNA or protein can be used to normalize data. Organ cultures are more difficult to normalize because of more heterogeneous cell populations, less well-defined culture conditions, and more difficult assessments of cell viability, but tissue wet weights can be used. Enzymatic dispersion techniques are available (25), but they add considerable time to the procedure and do not solve the problem of cell heterogenicity. Furthermore, if gonadotropin stimulation is required, highly purified enzyme preparations (i.e., collagenase) are necessary to avoid protese contamination and alterations in membrane-bound protein receptors (30). Hence, because of ease of culture, purity of cell type, and active basal steroidogenesis, isolated granulosa cell cultures, with or without added C_{19} androgen substrate, represent attractive models for evaluation of a potential toxicant's effect on steroidogenesis (Table 6).

Cell-cell interactions may control the pattern of ovarian steroidogenesis as evidenced by the so-called "spontaneous luteinization that granulosa cells undergo when placed in tissue culture independent of when they are harvested in the follicular phase (31). The removal of the cells from their approximation to the thecal layer, contact with follicular fluid, or disruption of intimate cell-to-cell contact appears to alter their steroidogenic potential and morphologic appearance *in vitro*. For these reasons the use of intact follicle walls without separation of the thecal and granulosa compartments may have to be considered as a test system if problems are encountered with isolated cell systems.

Selection of the species for use depends primarily on availability of adequate numbers of physiologically matured follicles or corpora lutea. While diethylstilbestrol-treated immature rats can be used as a source of ovarian cells (32), the numbers of cells are small and require a substantial time investment for collection. Domestic animals, by contrast, have much larger follicles, and the use of slaughterhouse material of a polyovulatory species minimizes the precollection time investment. Pigs and cows are the most desirable large animals to use in this regard, and both have an extensive literature available regarding their reproductive cycles, cell collection techniques, and tissue culture. The cell system chosen should be an easily exploitable model system in which known inhibitors of steroidogenesis in both human and animal systems can be studied *in vitro* to validate the animal model and the data extrapolated t humans for more general application to quantitative risk assessment of other suspicious compounds.

TABLE 6 Features of Ovarian Cell Preparations, *In Vitro*, Potentially Useful in Xenobiotic Inhibition of Steroidogenesis[a]

Characteristic	Separated		Intact	Luteal Cells	Stromal or Interstitial Cells
	Granulosa Cells	Thecal Cells	Follicle Walls (Thecal and Granulosa)		
Predominant steroids secreted (in order of amount)	Progesterone Estrogens[c] (only with added substrates)	Androgens[b] Estrogens Progesterone	Estrogen Androgens[b] Progesterone	Progesterone Estrogen	Androgens[b]
Steroid production independent of cycle stage	–	–	–	–	+
Steroid production not dependent on another cell type	–	+	–	+	+
Active basal steroidogenesis	+	+	+	+	–
Purity	+	–	NA	+	+
Ease of isolation	+	–	–	+[d]	+[d]

[a] A plus (+) indicates presence and a minus (–), absence of a characteristic listed in first column. NA means not applicable.

[b] Testosterone and androstenedione in varying proportion.

[c] Estradiol-17β and estrone in varying proportion.

[d] Dispersed cell culture requires enzymatic digestion; organ culture requires only excision.

REFERENCES

1. Gellert, R. J., Bakke, J. L. and Lawrence, N. L.: Persistent estrus and altered estogen sensitivity in rats treated neonatally with clomiphene citrate. Fertil. Steril. 22: 224–250, 1971.
2. Clark, J. H. and McCormack, S. A.: The effect of clomid and other triphenylethylene derivatives during pregnancy and the neonatal period. J. Steroid Biochem. 12: 47, 1980.
3. Eroschenko, V. and Palmiter, R.: Estrogenicity of kepone in birds and mammals. In: Estrogens in the Environment, J. McLachlan, Ed., Elsevier/North Holland: New York; pp. 305–326, 1980.
4. Clark, J. H. and Peck, E. J., Jr.: Female Sex Steroids: Receptors and Function: Springer-Verlag: Berlin; 245 pp., 1979.
5. Katzenellenbogen, J., Katzenellenbogen, B., Tatee, T., Robertson, D. and Landratter, S.: The chemistry of estrogens and antiestrogens. In: Estrogens in the Environment, J. McLachlan, Ed., Elsevier/North Holland: New York; pp. 33, 1980.
6. Kupfer, D. and Bulger, W.: Estrogenic properties of DDT and its analogs. In: Estrogens in the Environment, J. McLachlan, Ed., Elsevier/North Holland: New York; pp. 239–263, 1980.
7. Chan, L. and O'Malley, B. W.: Mechanism of action of sex steroid-hormones. I. N. Engl. J. Med. 294: 1322–1328, 1976.
8. Chan, L. and O'Malley, B. W.: Mechanism of action of sex steroid-hormones. II. N. Engl. J. Med. 294: 1372–1381, 1976.
9. Chan, L. and O'Malley, B. W.: Mechanism of action of sex steroid-hormones. III. N. Engl. J. Med. 294: 1430–1437, 1976.
10. Clark, J. H., Anderson, J. N. and Peck, E. J., Jr.: Estrogen receptor antiestrogen complex: atypical binding by uterine nuclei and effects on uterine growth. Steroids 22: 707–713, 1973.
11. Bliss, C. I.: Statistical methods in vitamin research. In: Vitamin Methods, P. Gyorgy, Ed., Academic Press: New York; pp. 448–609, 1951.
12. Pfaff, D. W.: Estrogens and Brain Function: Neural Analysis of a Hormone-Controlled Mammalian Reproductive Behavior. Springer-Verlag: New York; 272 pp., 1980.
13. Gerall, A. A. and McCrady, R. E.: Receptivity scores of female rats stimulated either manually or by males. J. Endrocrinol. 46: 55–59, 1970
14. McClintock, M. K. and Adler, N. T.: The role of the female during copulation in the wild and domestic Norway rat. Behavior 67(1–2): 67–96, 1978.
15. Mattison, D. R.: How xenobiotic compounds can destroy oocytes. Contemp. Ob. Gyn. 15: 157–169, 1980.
16. Mattison, D. R. and Thorgeirsson, S. S.: Smoking and industrial pollution and their effects on menopause and ovarian cancer. Lancet 1: 187–188, 1978.
17. Mattison, D. R.: Difference in sensitivity of rat and mouse primoridal oocytes to destruction by polycyclic aromatic hydrocarbons. Chem. Biol. Interact. 28: 133–137, 1979.

II. GLOSSARY OF TERMS USED IN FEMALE REPRODUCTION

adenosis—a nonneoplastic glandular disease that occurs in the uterine arnix and upper vagina.

amenorrhea—absence or abnormal cessation of the menses.

androgen—a class of steroid hormones produced in the gonads and adrenal cortex that regulate masculine sexual characteristics; a generic term for agents that encourage the development of or prevent changes in male sex characteristics; a precursor of estrogens.

androgen antagonist or **antiandrogen**—agent that opposes or impedes the action of an androgen.

anovulation—suspension or cessation of the escape of ova from the follicles.

corpus luteum—an endocrine body formed in ovary at site of ruptured Graafian follicle that secretes an estrogenic and progestagenic hormone.

diestrus—quiescent period following ovulation in the estrous cycle of female mammals in which the uterus prepares for reception of a fertilized ovum.

dopamine or **hydroxytyramine**—an intermediate in tyrosine catabolism and the precursor of norepinephrine and epinephrine.

ectopic pregnancy—pregnancy occurring outside the uterine cavity
egg—female sexual cell.

estradiol—an estrogenic hormone ($C_{18}H_{24}O_2$) produced by follicle cells of the vertebrate ovary; provokes estrus and proliferation of the human endometrium.

estrogen—estrogenic hormone; generic term for various natural or synthetic substances that produce estrus.

estrogen agonist—an agent that has a biological activity similar to that of the physiological estrogens.

estrogen antagonist or **antiestrogen**—agent that opposes or impedes the action of an estrogen.

estrus—phase of the sexual cycle of female mammals characterized by willingness to mate and in intact animals when ovulation occurs.

follicle (ovarian)—one of the vascular bodies in the ovary, containing the oocytes.

follicle-stimulating hormone or **FSH**—a glycoprotein hormone secreted by the anterior pituitary of vertebrates that promotes spermatogenesis and stimulates growth and secretion of the Graafian follicle.

galactorrhea—continued discharge of milk from the breasts in the intervals between nurshing or after weaning.

gonadotropin—a substance that acts to stimulate the gonads.

Graafian follicle—mature mammalian ovum with its surrounding epithelial cells.

gynecomastia—excessive development of the male mammary glands, sometimes leading to milk secretion.

hypothalamic-pituitary-ovarian axis—the hormonal interactions that link and control female reproduction.

hypothalamic-hypophyseal complex—the structural and hormonal relationships between the hypothalamus and the pituitary.

lactation—the production of milk; the period following childbirth during which milk is formed in the breasts.

luteinizing hormone or **LH**—glycoprotein hormone secreted by the adenohypophysis of vertebrates that stimulates hormone production by interstitial cells of gonads.

menopause—natural physiologic cessation of menustration, normally occurring in the last half of the fourth decade.

oligomenorrhea—prolongation of menstrual cycle beyond average limits.

18. Mattison, D. R. and Thorgeirsson, S. S.: Gonadal arylhydrocarbon hydroxylase in rats and mice. Cancer Res. 38: 1368–1373, 1978.

19. Mattison, D. R. and Thorgeirsson, S. S.: Ovarian arylhydrocarbon hydroxylase activity and primordial occyte toxicity of polycyclic aromatic hydrocarbons in mice. Cancer Res. 39: 3471–3475, 1979.

20. Mattison, D. R., West, D. M. and Menard, R. A.: Differences in benzo(*a*)pyrene metabolic profile in rat and mouse ovary. Biochem. Pharmacol. 25: 2101–2104, 1979.

21. Zuckerman, S.: The Ovary. Academic Press: New York; 600 pp., 1962.

22. Pedersen, T. and Peters, H.: Proposal for a classification of oocytes and follicles in the mouse ovary. J. Reprod. Fertil. 17: 555–557, 1968.

23. Armstrong, D. T. and Dorrington, J. H.: Estrogen biosynthesis in the ovaries and testes. In: Regulatory Mechanisms Affecting Gonadal Hormone Action, Advances in Sex Hormone Research, J. A. Thomas, and R. L. Singhal, Eds., University Park Press: Baltimore; 3: 217–258, 1977.

24. Stouffer, R. L., Nixon, W. E. and Hodgen, G. E.: Estrogen inhibition of basal and gonadotropin-stimulated progesterone production by Rhesus monkey luteal cells *in vitro*. Endocrinol. 101: 1157–1163, 1977.

25. Williams, M. T., Roth, M. S., Marsh, J. M. and LeMaire, W. J.: Inhibition of Human chorionic gonadotropin-induced progesterone synthesis by estradiol in isolated human luteal cells. J. Clin. Endocrinol. Metab. 48: 437–440, 1979.

26. Goldberg, V. J. and Ramwell, P. W.: Role of prostaglandins in reproduction. Physiol. Rev. 55: 325–351, 1975.

27. McNatty, K. P., Makris, A., DeGrazia, C., Osathanondh, R. and Ryan, K. J.: The production of progesterone, androgens and estrogens by granulosa-cells, thecal tissue, and stromal tissue from human ovaries, *in vitro*. J. Clin. Endocrinol. Metab. 49: 687–699, 1979.

28. Haney, A. F. and Schomberg, D. W.: Steroidal modulation of progesterone secretion by granulosa-cells from large porcine follicles: a role for androgens and estrogens in controlling steroidogenesis. Biol. Reprod. 19: 242, 1978.

29. Erickson, G. F., Wang, C. and Hsueh, A. J. W.: FSH induction of functional LH receptors in granulosa cells cultured in a chemically defined medium. Nature 279: 336–338, 1979.

30. Gulyas, B. J., Yuan, L. C. and Hodgen, G. D.: Progesterone production by dispersed monkey (*Macaca Mulatta*) luteal cells after exposure to trypsin. Steroids 35: 43–51, 1980.

31. Channing, C. P.: Influences of the *in vivo* and *in vitro* hormonal environment upon luteinization of granulosa cells in tissue culture. Recent Prog. Horm. Res. 26: 589–622, 1970.

32. Hall, P. F. and Young, D. G.: Site of action of trophic hormones upon the biosynthetic pathways to steroid hormones. Endocrinol. 82: 559, 1968.

oocyte—female ovarian germ cell present after birth.

ovarian cyclicity—the periodic changes observed in the ovary associated with follicular growth, ovulation, and corpus luteum function.

ovulation—discharge of an ovum or ovule from a Graafian folicle in the ovary.

parturition—labor; giving birth.

postpartum estrus—estrus with ovulation and corpus luteum production which occurs in some species immediately after birth of offspring.

progesterone—a steroid hormone ($C_{21}H_{30}O_2$) produced in corpus luteum, placenta, testes, and adrenals that plays a physiological role in the luteal phase of menstrual cycle and maintenance of pregnancy; also an intermediate in biosynthesis of androgens and estrogens.

prolactin—a protein hormone produced by adenohypophysis that stimulates secretion of milk and promotes functional activity of the corpus luteum.

prostaglandins—various 20-carbon-atom compounds, formed from essential fatty acids, that physiologically affect the female reproductive organs, the nervous system, and metabolism.

puberty—period at which the generative organs become capable of reproduction.

relative binding affinity—the degree to which a ligand, compared to standard ligand, is bound to a receptor.

secondary amenorrhea—any case in which the menses appeared at puberty but have been suppressed.

steroidogenesis—enzymatic steps converting acetate and cholesterol to sex steroids, glucosteroids, or mineralocorticoids.

testosterone—a biologically potent androgenic steroid which may be released from the gonads and adrenal glands.

virilization or **masculinization**—the assumption of male characteristics by a female because of excessive production of androgenic substances or masculinizing tumors of the ovaries.

CHAPTER 3

CONSIDERATIONS IN EVALUATING RISK TO MALE REPRODUCTION

INTRODUCTION

During evolution the reproductive patterns of mammals, including man, were determined to a considerable extent by the nature of the environment. Similarly today a variety of natural environmental factors may alter reproductive activity and fertility. Potential hazards to man's reproductive state are present in the environment as pollutants whose effects are often not likely to be clear-cut. Thus, sensitive assessment systems are needed. However, it is not clear how the potential effects on male reproduction can best be assessed.

Detailed information about many aspects of male reproduction exists, but there is little firsthand experience with detection in animals of subtle effects of either new chemicals or environmental hazards. The task of detecting effects that may be reflected only marginally in fertility performance is made more difficult by the variability of different reproductive parameters such as the concentration of sperm in an ejaculate, the total number of sperm ejaculated, and the sperm morphology within a population of "normal" men in our society. Hence, our understanding of the reasons for this variation and our ability to evaluate subtle responses to environmental hazards is minimal, and it is important to recognize the embryonic state of our abilities in this regard.

It is clear that changes in human reproductive function induced by environmental hazards might be reflected in reproductive behavior; in circulating levels of hormones such as follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone; or in testicular and epididymal function as evidenced by spermatogenic activity, fertilizing potential of the ejaculate, and ability of the sperm genome to support normal development after fertilization. Monitoring of a human population for the normality of any of these functions and assessment of the risk of certain levels of a chemical hazard require objective criteria that are measurable in man and, when a new chemical is to be evaluated, in an appropriate animal model. Ejaculates of human and of animal semen contain a considerable heterogeneity of spermatozoa, and several parameters of ejaculates may vary considerably from sample to sample. Nonetheless, there is now a reasonable knowledge of many facets of the normal physiology and of the variations to be expected for many specific parameters in animal models and to a lesser extent for man. The guidelines described below are based on current knowledge of the physiology of male reproduction in mammals, including man, and suggest an approach to risk assessment of existing and potential chemical hazards for reproductive function.

Aspects of the Problem

The single most sensitive and important parameter for human fertility is the total number of motile sperm in an ejaculate (1, 2). It has not been possible to set an exact limit on the minimal number of motile sperm per ejaculate, or what is more commonly reported as concentration of sperm or semen, necessary for fertility in man. A male ejaculating as few as 1×10^6 sperm per milliliter may prove fertile occasionally (3), but in most cases low numbers of sperm per ejaculate bear an obvious relationship to infertility. For example, sperm concentrations below 10 million, of from 10 to 20 million, and from 20 to 40 million per milliliter are associated with a risk of infertility that is, respectively, tenfold, fivefold, and threefold higher than for individuals with normal spermatozoal concentration, that is, 60 to 160 million per milliliter (4). Because another study (5) shows that relative risks are fourfold and twofold higher for men with sperm concentrations of below 10 million and between 10 and 20 million per milliliter, respectively, it is quite possible that a twofold reduction in sperm concentration in individuals with sperm counts below 40 million per milliliter will double the incidence of infertility. Several characteristics of human semen and testicular function reflect a low efficiency (2, 6). Human testes may function often at the threshold of pathology (2, 7, 8) and may be particularly sensitive to toxic agents compared with the testes of animals commonly used to study testicular function.

The yield of spermatozoa from spermatogonia, the rate of sperm production per gram of testis, and the percentage of morphologically normal sperm in ejaculates are lowest in man among the many mammals studied (2, 6–8). The median number of sperm ($\sim 200 \times 10^6$ per ejaculate) is only fourfold higher than the value (50×10^6 per ejaculate) below which fertility becomes significantly reduced (9). In contrast, the number of sperm in an ejaculate of bull semen (7×10^9), is 1400-fold higher than the value of 5×10^6 sufficient to achieve maximal fertility by artificial insemination (10), and a smaller animal, the rabbit, also shows a large differential. It is possible that a given set of conditions in the environment may cause infertility in man more readily than in experimental animals. Several agents, including radiation (11–14), chemotherapeutic drugs (15–18), and dibromochloropropane (19–22), reduce motile sperm concentrations and affect fertility.

Selection of an Animal Model

Evaluation of compounds for potential risk to human males requires one or more animal models. The selection and use of these models for testing end points that signify a reproductive hazard generally is more specialized than that for most toxicology or mutagenesis testing. The relevant end points depend on integrated functional aspects that can be monitored with ease only in certain species. The use of two species reduces the possibility of missing a hazardous agent during testing.

Parallel testing of both rat and rabbit seems most suitable. Although a number of laboratory or domesticated species might be used, rabbits and rats offer several advantages in comparison to dogs and subhuman primates. Rabbits have a high, predictable libido that may be useful in assessing risks to sexual behavior. More importantly, all sequential phases of the conception process (i.e., endocrine function, spermatogenesis, sperm maturation, ejaculation, sperm capacitation in the female, and fertilization) are easily evaluated, quantified, and manipulated throughout the year. The ability to characterize the whole ejaculate quantitatively and qualitatively and the ability to collect the ejaculate with ease using an artificial vagina make the rabbit a key test model for sensitive assessment of possible harmful effects of environmental agents on male reproduction.

The rat is also a very useful model and is preferable to the mouse or hamster because of the rat's widespread use in toxicological research, the large base of knowledge of its reproductive processes, its relatively low cost, its convenient size for weighing organs, and the fact that it breeds readily under laboratory conditions. The rat is less useful than the rabbit, because more of the measurements require invasive procedures and/or sacrifice of the individual. The characteristics of several potential models are summarized in Table 7 (1, 23).

TABLE 7 Criteria for Evaluation of Male Reproduction in Favored Animal Models and Man

	Mouse	Rat	Rabbit (New Zealand White)	Dog (beagle)	Monkey (rhesus)	Man
Duration of cycle of seminiferous epithelium (days)	8.6	12.9	10.7	13.6	9.5	16.0
Life span of						
B-type spermatogonia (days)	1.5	2.0	1.3	4.0	2.9	6.3
L+Z^a spermatocytes (days)	4.7	7.8	7.3	5.2	6.0	9.2
P+D^a spermatocytes (days)	8.3	12.2	10.7	13.5	9.5	15.6
Golgi spermatids (days)	1.7	2.9	2.1	6.9	1.8	7.9
Cap spermatids (days)	3.5	5.0	5.2	3.0	3.7	1.6
Fraction of lifespan as						
B-type spermatogonia	0.11	0.10	0.08	0.19	0.19	0.25
Primary spermatocyte	1.00	1.00	1.00	1.00	1.00	1.00
Round spermatid	0.41	0.40	0.43	0.48	0.35	0.38
Testes wt (g)	0.2	3.7	6.4	12.0	49	34
Daily sperm production						
Per gram testis (10^6/g)	28	24	25	20	23	4.4
Per male (10^6)	5	86	160	300	1100	125
Sperm reserves in cauda (at sexual rest; 10^6)	49	440	1600	?^{b}	5700	420
Transit time (days) through (at sexual rest)						
Caput + Corpus epididymides	3.1	3.0	3.0	?	4.9	1.8
Cauda epididymides	5.6	5.1	9.7	?	5.6	3.7

TABLE 7 (Continued)

	Mouse	Rat	Rabbit (New Zealand White)	Dog (beagle)	Monkey (rhesus)	Man
Evaluation possible of						
Testis size in situ	No	Yes	Yes	Yes	Yes	Yes
Number of testis spermatids	Yes	Yes	Yes	Yes	Yes	Yes
Testis histology	Yes	Yes	Yes	Yes	Yes	Yes
Quantitatively collected semen	No	No	Yes	Yes	Yes[c]	Yes
Feasible fertility tests						
Natural mating	Yes	Yes	Yes	No	No	No
Artificial insemination	No	No	Yes	No	No	No
In vitro	Yes	Yes	Yes	Yes	Yes	Yes
Analysis of seminal plasma for agent	No	No	Yes	Yes	Yes	Yes
Sufficient sperm for *in vitro* testing	No	No	Yes	Yes	Yes[c]	Yes
Sufficient blood to assay 3 hormones	Yes	Yes	Yes	Yes	Yes	Yes
Longitudinal hormonal analyses	No	Yes	Yes	Yes	Yes	Yes

[a] L = leptotene, Z = zygotene, P = pachytene, D = diplotene.

[b] A question mark indicates unclear or inadequate data.

[c] Semen obtained by electroejaculation.

Source: Adapted from Refs. 1 and 23.

Tests for Evaluating Reproductive Damage

We reviewed a wide spectrum of test systems. Table 8 (24–56) lists tests that were considered to be suitable for qualitative and quantitative risk assessment (for detailed consideration, see the Appendix to this chapter). Table 9 (including Refs. 57–70) lists additional tests that were considered but rejected because they are insensitive, redundant, not cost effective, too difficult to perform unless within a research setting, not validated, too controversial, or in need of further development.

The tests in Table 8 evaluate the endocrine control of male reproduction and the number and quality of sperm produced, or they measure fertility. Since fertility is related to the number of normal spermatozoa in the ejaculate, the analyses of seminal quality are indirect measures of fertility. However, comprehensive seminal analysis is a much more sensitive end point for detection of a toxic effect than a breeding experiment using natural mating, because in experimental animals the number of sperm ejaculated greatly exceeds the number necessary for fertility (10, 71). The various tests are stratified into a sequence (tests 1–3, S, E) ranging from a preliminary screen to more detailed studies.

Coefficients of variation (CV) might be used to determine the sensitivity of a study that compares treated animals with controls (see Appendix). Generally, measurements of testicular and epididymal sperm numbers give highly reproducible values in control animals with coefficients of variation between animals of 15% or less (24). This degree of reproducibility ensures that the test will be quite sensitive, even with relatively few animals (Table 10). Although there is appreciable variation (CV ≅ 70%) in sperm concentration or total number of sperm per ejaculate in semen collected from rabbits or bulls (72, 73), this variation can be reduced somewhat by using a uniform interval between seminal collections and standardized procedures in chronic studies. Sperm motility and morphology are much more constant than sperm number or concentration, especially within individuals of a species (10, 74). These two assays usually are performed in a subjective manner, and efforts must be made to minimize this subjectivity (2). In some species motility can be evaluated objectively by measurements made on time exposure negatives (75) or probably better on videotape recordings (49). Morphology of spermatozoa from individuals always should be compared to control samples analyzed concurrently by the same observer. The slides and/or videotapes should be retained for validation by an outside observer.

TABLE 8 Tests Considered Useful for Screening Toxic Compounds[a]

Test	Rat	Rabbit	Human	Reference
Body Weight	1–3	1–3	S, E	
Testis				
Size in situ	1–3	1–3	S, E	24–26
Weight	1–3	1–3	NP	24–27
Spermatid reserves	1–3^{b}	1–3^{b}	NP	24, 25, 27–30
Gross histology	1–3^{c}	1–3^{c}	NP	26, 31–34
Nonfunctional tubules (%)	$2,3^{b}$	$2,3^{b}$	NP	13, 18, 35
Tubules with lumen sperm (%)	2,3	2,3	NP	26, 32, 33
Tubule diameter	2,3	2,3	NP	26
Counts of leptotene spermatocytes	1–3	1–3	NP	32, 34
Epididymis				
Weight of distal half	1–3	1–3	NP	25, 27
Number of sperm in distal half	1–3	1–3	NP	27, 36, 37
Motility of sperm, distal end (%)	1–3	1–3	NP	38–40
Gross sperm morphology, distal end (%)	1	1	NP	41, 42
Detailed sperm morphology, distal end (%)	2,3	2,3	NP	6, 41, 42
Gross histology	NA	NA	NP	
Accessory Sex Glands				
Weight of vesicular glands	1–3	NA	NP	26, 27, 43
Weight of total accessory sex glands	NA	1–3	NP	26, 27, 43
Semen				
Total volume	NP	1–3	E	2, 24, 26, 44–46
Gel-free volume	NP	1–3	NA	24, 26, 44–46
Sperm concentration	NP	1–3	E	2, 24, 26, 44–46
Total sperm/ejaculate	NP	1–3	E	24, 26, 44–48
Total sperm/day of abstinence	NP	1–3	E	2, 24, 26, 48
Sperm motility, visual (%)	NP	1–3	E	2, 26
Sperm motility, videotape (% and velocity)	NP	2,3	E	2, 49
Gross sperm morphology	NP	1	NA	2
Detailed sperm morphology	NP	2,3	E	2, 42, 50
Concentration of agent in sperm	NP	NA	NA	
Concentration of agent in seminal plasma	NP	3^{d}	E^{d}	
Concentration of agent in blood	NP	3^{d}	E^{d}	
Biochemical analyses of sperm/seminal plasma	NP	NA	NA	2, 46, 51
Endocrine				
Luteinizing hormone	2,3	2,3	E	52, 53
Follicle-stimulating hormone	2,3	2,3	S, E	52, 53
Testosterone	2,3	2,3	E	52, 53
Gonadotropin-releasing hormone	2,3	2,3	E	52, 53
Fertility				
Ratio exposed: pregnant females	1–3	1–3	NP	54
Number embryos or young per pregnant female	1–3	1–3	NP	54
Ratio viable embryos: corpora lutea	1–3	NA	NP	54
Ratio implantation: corpora lutea	1–3	NA	NP	54
Number 2–8 cell eggs	3^{b}	NA	NP	55
Number unfertilized eggs	3^{e}	NA	NP	55
Number abnormal eggs	3^{e}	NA	NP	55
Sperm per ovum	3^{e}	NA	NP	55
Number of corpora lutea	3^{e}	NA	NP	
In Vitro				
Incubation of sperm in agent	NA	3^{f}	E^{f}	
Hamster egg penetration test	NA	NA	E	56

TABLE 8 (Continued)

[a] Test 1 = initial at maximum tolerated dose (MTD), or MTD and 0.7 MTD, run for exactly six cycles of the seminiferous epithelium. A similarly significant change (probably >15%) in any criterion would be evidence of an effect.

Test 2 = dose response at MTD, at −1 and −2 log dose, and down to human level if known or until no response is obtained in any test; run for exactly six cycles of the seminiferous epithelium.

Test 3 = long term, reversibility, several doses and time periods. Expose to at least three doses for at least 6 cycles of the seminiferous epithelium (kill 1/3 of males) and then allow recovery for 6 cycles (kill 1/3 of males) and 12 cycles (kill 1/3 of males). Recovery at 12 cycles after termination of treatment should be to at least 90% of control level to show complete reversibility.

S = procedure useful for screening humans in industrial setting.

E = procedure useful for studying individuals thought to be exposed to an agent. Evaluation of human semen should use samples obtained after 2 to 5 days of abstinence with samples taken over time.

NA = not necessary.

NP = not practical or possible.

[b] Especially important when studying recovery.

[c] Save tissue from level 1 test, fix in Bouins, for possible later use.

[d] In 3 samples taken near end of treatment and then in additional samples to get clearance rate.

[e] Female rats killed 18–24 hours after mating to evaluate fertility, sperm penetrating ability, and sperm transport.

[f] If compound is detected in seminal plasma of rabbit or man, incubate both rabbit sperm and human sperm from normal donors and determine a dose response of sperm to the drug *in vitro*. Evaluate percentage of motile sperm over time at 37° or *in vitro* penetration of hamster oocyte.

All of the tests listed in Table 8 are feasible in most well-equipped laboratories. The phase-contrast microscope and video micrography equipment (estimated additional cost: $6000) are the only nonstandard requirements. The training period necessary to conduct the tests in an accurate and precise manner is not excessive.

The tests selected can be used to (a) detect an effect of a test compound on male reproduction and (b) serve as a basis for estimating an acceptable level of exposure.

The tests listed will yield quantitative data that are amenable to efficient statistical analyses and that have a sufficient range of values to enable establishment of dose-response curves. The variability of the tests is shown in Table 10 for most parameters.

The proposed tests are for the most part quite specific for reproductive toxicity. Results of each test should not be affected by other body systems or, except for a possible decrease in testosterone level, cause changes in other aspects of body function.

Any subchronic or chronic test used to evaluate effects of an agent on male reproduction must extend over 6 cycles of the seminiferous epithelium, when it is assumed that an agent bioaccumulates to a steady state within 1 cycle (23). This interval is based on (a) the time needed to reach a steady state concentration of

TABLE 9 Reasons for Rejection of Potential Evaluation Tests Considered by Male Reproductive Subgroup

Test	References	Reasons for rejection[a]
Tonometric measurement of testicular consistency	26, 57	US (rat), NV (rabbit), FR (human)
Qualitative testicular histology	24, 31, 34	I
Stage of cycle at which spermiation occurs	24, 31, 34	RR, UR
Quantitative testicular histology		
Counts of degenerating germ cells	18, 35, 58	UR
Complete germ cell counts	18, 35, 58	UR, $$
Stem cell counts	18, 35, 58	UR, $$, FR (rabbit)
Relative frequency of stages of cycle	18, 35, 58	UR, I
Epididymal histology	59, 60	I, NR
Biochemistry of epididymal fluids	2, 61	NR
Histology of accessory sex glands	62	NR
Biochemical analysis of sperm	2	NR, $$
Sperm membrane characteristics	63, 64	NV, FR
Biochemical analysis of seminal plasma	65	NR
Evaluation of sperm metabolism	65, 66	NR, $$
Fluorescent Y bodies in spermatozoa	67, 68	NV, NR
Flow cytometry of spermatozoa	69	UR, NV, FR
Karyotyping human sperm pronuclei	70	FR
Cervical mucous penetration test		UR (human)
Studies on prepuberal animals		NR (rat, rabbit)[b]

[a] US = unsuitable for species
NV = not validated
RR - redundant
I = insensitive
NR = not relevant
UR = only in specialist lab
FR = future research
$$ = too costly

[b] Studies on animals treated prior to puberty have not been included for the following reasons. It would involve a redetermination of maximum-tolerated-dose levels for young, growing animals. The choice of age period of exposure is a complex topic and sufficient time was not available to adequately consider this. Humans are exposed to many of the agents that would cause reproductive problems primarily through occupational exposure after puberty. Some agents (radiation, cyclophosphamide) that cause reproductive problems with prepuberal exposure also affect postpuberal males. Nonetheless, unique developmental processes occur in testicular development prior to and during puberty, and therefore a possibility exists that some agents would only affect the prepuberal male. The group of tests proposed in Table 8 would provide a sensitive measure of such effects, if animals exposed at any time during puberty or throughout their development were analyzed after reaching sexual maturity.

agent in the target organs of the rabbit or rat, (b) the concepts that an agent acting directly or indirectly on the germinal epithelium may act on a specific type of cell and that affected germ cells may develop for some time before they degenerate, (c) the fact that damage to germ cells is most evident by absence of certain *types* of germ cells, and (d) qualitative change in germ cells may not be readily discernible until active spermatozoa pass into the cauda epididymidis or ejaculated semen. The present protocol assumes that attainment of a steady state concentration of an agent requires an interval equal to one cycle of the seminiferous epithelium. Formation of primary spermatocytes from renewing spermatogonia in the

TABLE 10 Approximate Variation Between Animals for Suggested Test Criteria (CV)[a] Coefficient of Variation (%)

Criterion	Rat model[b] (Wistar)	Rabbit model[c] (New Zealand White)	(Dutch Belted)
Body weight	20	37	9.5
Testis			
Weight	5	18	20
Size in situ	NA[d]	9	20
Spermatid reserves per testis	11	24	28
Spermatid reserves per gram	8	9	
Tubule diameter	–[e]	5	11
Epididymis			
Weight of distal half	13	13	20
Number of sperm in distal half	20	52	30
Motility of sperm, distal end (%)	–	–	12
Gross sperm morphology, distal end (%)	–	–	
Detailed sperm morphology, distal end (%)	–	–	8
Accessory sex glands			
Weight vesicular glands	26	100[f]	100[f]
Weight total accessory sex glands		25	
Semen			
Total volume	NA	–	50
Gel-free volume	NA	40	50
Sperm concentration	NA	41	60
Total sperm per ejaculate	NA	–	75
Total sperm per day of abstinence	NA	28	–
Sperm motility, visual (%)	NA	–	12
Sperm motility, videotape (%)	NA	–	–
Gross sperm morphology	NA	–	–
Detailed sperm morphology	NA	–	–
Concentration of agent in seminal plasma	NA	–	8
Concentration of agent in blood	NA	–	–
Endocrine			
Luteinizing hormone	80		
Follicle-stimulating hormone	65		
Testosterone	33		
Gonadotrophin-releasing-hormone stimulation	–		
Fertility[f]			
Ratio of exposed to pregnant females			15
No. embryos or young per exposed female			20%
No. embryos or young per pregnant female			15%
Ratio of embryos to corpora lutea			10%

[a] Data are not available to allow calculation of sensitivity of the tests used with humans.
[b] Ref. 27.
[c] Refs. 25, 37, and 44.
[d] NA = not applicable.
[e] – = data not available.
[f] For controls: with treatment, variability may be greater.

rat requires about 1.5 cycles, and spermiation occurs about 3 cycles of the seminiferous epithelium after those sperm have become primary spermatocytes. Passage of sperm through the epididymis into the distal cauda or ejaculated semen requires 1.0 to 1.5 cycles, depending on the species and frequency of ejaculation. Consequently, if an agent acted on A-type spermatogonia, a decrease in number of sperm ejaculated or in the fertility of sperm from the cauda epididymidis might not occur for 5 to 6 cycles (1.5 + 3.0 + 1.0 = 5.5 cycles) of the seminiferous epithelium. If the agent resulted in degeneration of pachytene spermatocytes, an alteration in semen characteristics or fertility might be expected to occur after 4.0 to 4.5 cycles. However, with continuous exposure to the test compound, such a lesion would remain detectable in the semen or by examination of testicular histology at the end of 6 cycles of the seminiferous epithelium.

By testing male rats or rabbits for their fertility after 5 cycles, a depression in fertility caused by a compound inducing a qualitative change in sperm function should be detectable, since this probably would affect spermatocytes or spermatids. Allowing 6 to 8 days of sexual rest between the end of fertility testing and necropsy of test males after 6 cycles provides time for restoration of the normal population of sperm in the cauda epididymidis in males receiving doses that do not suppress daily sperm production. If sperm production is low in test males, the reserve level in the cauda will reflect this, but sufficient sperm may still be present to allow assessment of sperm motility and morphology.

For these reasons, evaluation of an agent, administered chronically, for effects on male reproduction should include fertility tests after 5 cycles and examination of the testes, epididymides, accessory sex glands, and plasma hormone levels after 6 cycles of the seminiferous epithelium.

If an agent is shown to alter male reproduction in a test extending over 6 cycles of chronic exposure (Test 1 or Test 2 as described under protocols for testing), it may be desirable to determine if the effect is reversible (Test 3). A test of reversibility should extend over 18 cycles of the seminiferous epithelium. Chronic exposure to the agent should extend over 6 cycles, and 12 cycles should elapse after the termination of exposure to allow for restoration of normal reproductive function.

Although the recovery period in man is usually longer, these animal data should provide a clear indication if complete recovery will occur in man (compare radiation data of Meistrich et al. [13] in the mouse and Rowley et al. [14] in man).

Evaluation of Reproductive Damage in Exposed or Potentially Exposed Men

General

Two types of studies in humans seem particularly relevant to the objectives of a male reproduction risk assessment. The first involves surveillance studies, in which periodic checking is done on men in a setting (e.g., industrial or agricultural) that might, in the future, involve the risk of a reproductive defect. An example of such a setting would be a chemical company in which substances ar prepared that are known from animal studies to cause reproductive toxicity when administered in high dosage but not at a dosage up to 10 times that expected for human exposure. The safety factor may be variable, however, and depends on the quality of the animal study from which it was derived. This type of surveillance is important for at least two reasons: (a) the sensitivity of men may be greater than allowed for by the tenfold safety factor and (b) the exposure of the workers may be greater than originally estimated. The methods for surveillance of this type could be quite innocuous and could be incorporated into an annual medical checkup if this were already a practice.

The second type of study would be of men who have been or are being exposed to a known reproductive toxin in dosages likely to be toxic in man as based on animal studies. This type of study could be used in men exposed to high dosages of one or more general toxins for which the effect on testicular function had not been studied carefully in animals.

Surveillance studies

Men could be asked yearly whether they have been attempting to cause a pregnancy and have been unable to do so. The prevalence of infertility in couples within the reproductive age group is approximately 15% (74). If more than 20% of men between 19 and 35 hav been unable to produce a pregnancy in over one year of *unprotected* intercourse, a possible toxic effect should be looked for in a rigorous manner as outlined in the following section on known toxic exposure.

Testicular length could be measured on annual physical examination. If the distribution of testicular size for men falls significantly below the lower norm (3.5 cm for Caucasian and Black) for that age group and ethnic background (76), a toxic effect should be suspected.

Blood levels of follicle-stimulating hormone could be measured yearly. If mean levels significantly vary from those of age-matched controls, a toxic effect should be suspected.

If any of these three variables suggest testicular toxicity, a more detailed study of the population should be undertaken, as outlined below.

Study of men with known toxic exposure

Where a human population is suspected of being at reproductive risk because of environmental hazards, a number of potentially toxic agents may be involved, and the duration and level of exposure may vary within the population. Although each potential toxicant should be carefully tested by the laboratory screening methods outlined in this document, it would be useful to make a more immediate and direct assessment of fertility potential in the exposed population. The requirements for this include capacity for rapid response to the subjects, feasibility, sensitivity, and data that can be analyzed statistically. The data obtained in such studies should provide an initial indication of the degree of testicular damage, and where an environmental reproductive hazard has been identified, more detailed studies may be undertaken to characterize objectively male reproductive dysfunction.

To carry out these studies, a specialized team and a modest amount of equipment would be needed. The latter could be installed at locally available facilities, or a mobile laboratory could be equipped. Detailed medical, reproductive, and occupational histories should be taken from each exposed subject and a physical examination given. At least five semen samples should be evaluated per individual at two-day intervals. Objective data on testicular size and consistency could be obtained by sonography and tonometry. Blood and urine could be obtained at this time for endocrine studies and/or toxicant levels. Controls to be studied must be carefully chosen and matched. Before a national data base is established, individuals should be selected according to epidemiological advice. For the details of these analyses, see "Study of men with known toxic exposure" in the Appendix to this chapter.

Statistically significant differences between the exposed and the control groups (matched for age, occupation, geographical location) in seminal fluid and blood hormone measurements would be evidence for an effect of the exposure on male reproductive function. An adverse effect would be expected to decrease sperm counts, motilities, and numbers of sperm with normal morphology; if the effect were sufficiently severe, blood testosterone levels would decrease. If the toxic effects were directly on the testis (as is the case with the great majority of known toxins), follicle-stimulating hormone (FSH) levels would increase. With a mild toxic effect on the testis, blood FSH levels after administration of gonadotropin-releasing hormone (GnRH) might exceed normal responses, even when basal FSH levels are normal. If the toxic effect were primarily on the pituitary gland or central nervous system, luteinizing hormone (LH) and FSH levels would tend to decrease.

If not established initially (Surveillance studies, v. sup.), oth comparisons between the exposed and control groups should include (a) rate of infertility as indicated by the number of men who have not been able to induce a pregnancy in over one year of intercourse without using contraceptives, and (b) testicular size, with particular attention to the number of men with testicular length less than 3.5 cm.

Differences between the exposed and control groups in these last two assessments, suggested also for the initial screen, will be found when reproductive toxicity is sufficiently severe. However, measurements of fertility and testicular size would be expected to be less sensitive in revealing mild defects in gonadal function than the seminal fluid and blood hormone measurements described above.

Additional comment on human testing procedures

Blood samples for hormone measurement and noninvasive procedures such as testicular length may be the more feasible parameters to evaluate because of the added difficulty in obtaining semen samples, in some human populations at least. However, where these give equivocal results, it is likely that semen analyses will help to resolve the fundamental dilemma.

An elevated FSH level is a sensitive indicator of decreased function of the germinal epithelium in man and experimental animals. However, while there is no doubt that increased FSH lev usually imply decreased sperm production (9, 14, 21, 77, 7 measuring FSH levels is probably a less dependable test than direct sperm counts, for it is a consistent marker only of severe oligozoospermia or azoospermia (53, 79, 80). Measurements of FSH seem useful adjuncts to sperm counts, therefore, and indicators of the direct action of toxic agents on pituitary function. Despite their wide variability in man, total sperm per ejaculate are usually a *more sensitive* measure of testicular damage than elevated FSH levels.

Comprehensive semen analysis requires an assessment of sperm morphology as well as total sperm per ejaculate. This aspect of the human ejaculate has received considerable general comment as a parameter that also often falls below the standards that might be expected for animals living in the same area. Since about 30% of the spermatozoa are abnormal in semen from a presumed fertile group of men, only by using large groups of 100 or so persons would it be possible to detect increases in abnormal spermatozoa of the order of 10% in cross-sectional studies in which only one to four samples are collected for each man in exposed and control groups. Despite the relatively variable morphology of a significant proportion of spermatozoa in the human ejaculate, sperm morphology tends to be fairly constant for one individual (81). This justifies the use of fewer men in longitudinal studies where such studies can be undertaken (as compared to postexposure analyses), since the men can then act as their own controls (81).

Human sperm morphology classification is currently subjective, personality oriented, and nonstandard (82). However, detailed "type classification" (i.e., oval versus tapering versus amorphous) may not be required to identify groups of individuals at reproductive risk. In normal fertile human semen, sperm morphology is relatively uniform, more than 50% of the sperm having the typical "oval" shape. In contrast, infertile human semen is characterized by a diversity of abnormal sperm sizes and shapes. If objective, morphometric data describing sperm size and shape (e.g., head length, width, area, and circumference; tail midpiece width) were obtained from individuals at potential risk, these could be compared statistically with data from the matched control group. Significantly greater dispersion in the morphometric parameters of the exposed group might indicate increased reproductive dysfunction in the population. The magnitude of differences between the exposed group and the control group might also provide an indication of the severity of testicular damage. As noted earlier, the methods for automatic evaluation of sperm morphology are not well established and need considerable refinement and validation (6).

Assessment of risk to men

Assessment of risk to reproductive performance and fertility in men is inadequately tested at present. The quantitative assessment of risk to general human health from exposure to environmental toxicants has been approached by relating the probable or estimated dose of a suspect toxic agent to the occurrence of deleterious effects on the basis of either epidemiologic data on human populations or of experimental data from animal studies. It seems likely that threshold effects will appear for most agents. Ideally, assessment of the effects of these agents upon male reproductive function should be based upon human epidemiologic data. However, there are few epidemiologic risk assessment data regarding the effect of environmental agents on the fertility of men. Thus, at present a quantitative risk assessment must depend on extrapolation to man of measurements of the reproductive end points in experimental animal systems discussed here. One approach to risk assessment estimates the acceptable daily intake (ADI) of a chemical, defined as the exposure level that is anticipated to be without risk to the species. It should be cautioned that the ADI represents only a judgment, is not an estimate of risk nor a guarantee of absolute safety, and is subject to modification as additional relevant information becomes available.

To account for the uncertainties involved in extrapolating from animals to man, the ADI includes an uncertainty or safety factor to the highest no-adverse-effect level measured in an animal study. A no-adverse-effect level is defined here as a dose for which no significant difference is found between control and treated animals for any of the end points measured adequately. It is important that a statistically significant effect also be biologically significant. This uncertainty factor will depend on (a) the animal species/strain; (b) the quality of the experimental data; (c) the availability of comparative pharmacokinetic information on the animal species' and man's absorption, distribution, metabolism, binding, and elimination of the chemical; and (d) any other relevant comparative information on structurally similar chemicals. In the absence of these comparative data, we should follow the guidelines of the Safe Drinking Water Committee, National Research Council of the National Academy of Sciences (83), and recommend an arbitrary uncertainty factor of 100 for adequate animal studies. In the case of human male reproduction, the size of this factor seems more than justified by increasing evidence that the human testis functions less efficiently and possibly closer to a point of pathology than that of the animal models recommended (2, 7, 8). Thus, for an agent causing a reversible action in a model animal, the ADI would be 0.01 times the no-adverse-effect level for the most sensitive criterion and the most sensitive species evaluated, whether rat or rabbit. A daily exposure or intake above this level represents a risk of reproductive damage to human males. For irreversible effects on male reproductive function, we feel we can make no recommendation for a quantitative risk assessment.

Protocols for Testing Compounds with Animal Models

The actual criteria to be evaluated in each test are shown in Table 8. The time schedules for conducting Tests 1, 2, and 3 are shown in Table 11. If pharmacological studies show that the test compound may bioaccumulate so that the body burden increases beyond an interval equal to one cycle of the seminiferous epithelium, the treatment interval of both Test 1 and Test 2 must be increased appropriately. At least 5 cycles should elapse after reaching maximum body load.

Test 1 – initial screen

As an initial screening procedure, animal exposure will be greater than or equal to half of a maximum tolerated dose (⩾0.5 MTD) of the test agent for an interval equal to 6 cycles of the germinal epithelium. An initial screen using an acute exposure is considered to be unnecessary, because the subchronic test is more sensitive.

To initially assess risk to male reproduction, a compound should be subjected to *in vivo* tests utilizing both rats and rabbits. A compound producing no statistically significant alteration in any criterion for either species when given at ⩾0.5 MTD would be considered to be safe for humans (see safety factor in risk assessment). A statistically significant alteration in any criterion would necessitate conduct of a Test-2 evaluation to establish a dose-response curve, unless manufacture or use of the agent were to cease, or if a larger safety factor were used. Test 1 (Tables 8 and 11) uses both rats and rabbits and is detailed in the Appendix to this chapter. The fixed time schedule is designed to maximize the probability of detecting any decrease in reproductive function.

Test 2 – dose response curve

1. The general approach used in the initial screen (Test 1) will be used except that additional criteria of reproductive damage are included (Table 7). The dose-response curve will include at least three points, usually the dose used in Test 1, and –1 and –2 log doses and must extend down to the human exposure level (if known) or until no statistically significant response is obtained in any test. Both 0-dose and untreated controls could be included to detect effects of handling that might be associated with agent administration. If necessary, additional tests will be run to attain these end points. Both rats and rabbits must be used. The fixed time schedule (Table 11) is essential to measure accurately the extent of damage to

TABLE 11 Chronology of Conduct for Test with Animal Models (Expressed as Day of Study)

Test	Rat	Rabbit
Test 1 or 2[a]		
Condition males	−21 → 0	−28 → 0
Obtain preexperimental body weight	−14 + 0	−14 + 0
Evaluate preexperimental semen	NA[b]	−14 → 0
Initiate compound administration	day 0	day 0
Continue compound administration	0 → 77	0 → 64
Weigh weekly	0 → 78	0 → 65
Collect experimental semen (each 3–4 days)	NA	3–4 → 53–54
Measure testis size weekly	NA	0 → 65
Expose to females or artifically inseminate females	65 → 71	54 → 57
Sexually rest males	71 → 78	58 → 65
Kill males	78	65
Kill females	83 → 89[c]	NA
Allow females to kindle	NA	85 → 90
Test 3[a]		
Condition males	−21 → 0	−28 → 0
Obtain preexperimental body weight	−7 + 0	−7 + 0
Evaluate preexperimental semen	NA	−14 → 0
Initiate compound administration	0	0
Continue compound administration	0 → 77	0 → 64
Collect experimental semen	NA	35 → 64
	NA	118 → 140
	NA	158 → 194
Expose to females or artifically inseminate females	215 → 221	184 → 187
Kill 1/3 of males	78	64
1/3 of males	155	128
1/3 of males	232	193
Kill females 18–24 hours past mating	216 → 222	NA
12–18 days past mating	233	NA
Allow females to kindle	NA	215 → 218

[a] Rats will be weighed weekly. Rabbits will be weighed weekly, testis size measured weekly, and semen will be collected twice weekly (every 3 to 4 days). Schedule is for a compound that does not accumulate for a long time; steady state level in body tissues reached in <10–12 days.

[b] NA = not applicable.

[c] Kill females 18 days after mating, as determined by a vaginal smear.

the different aspects of male reproductive function and to enable a prediction of human risk.

2. In evaluating testicular histology, sections representing at least two loci will be used. The diameter of 50 tubules will be measured; the percentage of seminiferous tubule cross-sections (N = 250) having mature spermatids lining the tubule lumen and the percentage of

tubules (N = 250) devoid of germ cells other than spermatogonia will be determined. Evaluations of the morphology of sperm in the cauda epididymidis and ejaculated semen will be more comprehensive than in Test 1. The serum concentrations of luteinizing hormone (LH), FSH, and testosterone also will be determined.

Test 3 – recovery study

1. Test 3 is a long-term study designed to test the reversibility of damage to male reproduction and also to evaluate sperm transport, penetration of sperm into ova, and early embryonic death. Many agents that cause degeneration of the germinal epithelium and azoospermia will not damage the stem spermatogonia. If the latter remain, eventual recovery of the germinal epithelium is likely. This test measures recovery of the germinal epithelium and fertility, at 6 cycles and 12 cycles (155 days for rats and 128 days for rabbits) after ending a 6-cycle exposure to the test compound. Although recovery of the germinal epithelium might not be complete by 12 cycles after exposure, some onset of recovery probably should be detectable by then if it will occur eventually. If the test compound is one known to bioaccumulate, longer treatment periods (as used in Test 2) and recovery periods (at least twice the duration of the treatment period) are essential.

2. The criteria evaluated (Tables 8 and 11) are the same as those in Test 2, except that data on fertilized rat eggs are necessary. Consequently, each male will be exposed to four female rats. Two females will be killed 18 to 24 hours after mating (as determined by the presence of vaginal plugs) and ova recovered by flushing. The two other female rats will be killed 12 to 18 days after mating. Measurement of concentrations of the compound in blood and seminal plasma at steady state are desirable, since these data may be useful in predicting potential damage in humans and the prognosis for recovery from such damage.

3. The time schedule (Table 11) for conduct of the study could be modified by extending the treatment beyond 6 cycles, but the timing of evaluations between days 215 and 233 for rats and days 184 and 218 for rabbits may not need to be altered.

4. Complete reversibility is considered to be restoration, to at least 90% of control levels at 12 cycles after cessation of exposure, of *all* criteria adversely altered in males after 6 cycles of exposure (in Test 2 or Test 3) at a given dose.

Research Needed

Until about 15 years ago only outline information about the male tract was available (84–86). Although much precise data has appeared since then for the animal models suggested and even some for man (65, 87, 88), it is difficult to compare the two. Research into this and related aspects as suggested below is a critical element for establishment of a reliable assay and evaluation of risk in males.

1. Variance components for characteristics of semen (total volume, total sperm per ejaculate, percentage of motile sperm, and incidence of sperm abnormalities) are available for rabbits (see Table 10) but have not been reported for man. This information should be obtained for men of different age groups (<20, 21–30, 31–40, >40) with different life styles or occupations, so that efficient and meaningful evaluations can be made.

2. The influence of an abstinence interval on characteristics of human semen should be evaluated critically for men of 20 to 30, 30 to 40, and >40 years of age. Procedures for reducing the influence of an abstinence interval on estimates of sperm production (e.g., normalization of data for each ejaculate by dividing by the number of days of abstinence) should be evaluated. It is also unclear what effect repeated ejaculation has on the absolute concentrations of many seminal compounds that might be measured as indicators of the activity of accessory glands in man or the amount of the test agent in seminal plasma.

3. For the human, the relationship should be determined among testicular size, tonometric measurements or testicular consistency, and sperm output as well as other ejaculate characteristics.

4. Relationships need to be established among testicular histology, ejaculate characteristics, sperm morphology, and fertility of humans, rabbits, and rats. Indices of fertility should be calculated.

5. Automatic or semiautomatic morphometric procedures should be further developed for analysis of the morphology of human spermatozoa and spermatozoa from test animals. First-generation systems for automated evaluations are available (e.g., at Lawrence Livermore Laboratory), but the instrumentation and software need additional refinement and validation before these techniques can be applied routinely in analyses of human sperm morphology (89, 90).

Sperm morphometry can be obtained for living sperm cells or from stained seminal smears, by methods becoming increasingly automated, either using flow cytometry (89) or tracing sperm shape from the screen of a video monitor using an electronic planimeter-digitizer integrated into a minicomputer (49). The use of sperm morphology for diagnosis or prognostication of specific reproductive disorders will require a "type classification" of individual sperm abnormalities. The determination of such a classification can be made by computer on the basis of the morphometric data obtained. Research should be encouraged to develop such computer software. A classification system based on morphometric standards should be developed.

6. A data bank should be established for (a) the control data from screening tests with animals to build a large base for computation of variations associated with each characteristic among trials, locations, season, year, etc.; (b) the chemical nature of compounds tested and found in an initial screen to damage some aspect of male reproduction or to have no effect; and (c) the chemical nature of all compounds found to have a deleterious effect on male reproduction, the nature, extent, and incidence of damage in each exposure dose, and interval to recovery.

7. Available data on effects of agents known to alter human male reproduction should be correlated with data on their action in test animals in a battery of tests. The repeatability and relative sensitivity of the tests within and between species should be determined. Recommendations for specific studies are as follows:

(a) Obtain more extensive and accurate analytical data on semen from men exposed to chemotherapy and the effects of parallel levels of chemotherapy in animals.

(b) Bring together existing radiation studies in human and experimental animals for development of models in which tc base chemical risk assessment data.

(c) Obtain better data in experimental animals on effects of dibromochloropropane or other agents known to be harmful to man.

8. The relative usefulness of basal FSH concentration in blood or of FSH response to GnRH as indicators of testicular damage should be compared with seminal analyses to determine their sensitivity (see Table 10 and Appendix to this chapter). For screening of large numbers of human males, it would be useful to know the single most sensitive index of testicular toxicity. In man, a test based on a blood sample is more practical than one requiring submission of a seminal fluid sample.

9. The responsiveness of Leydig cells should be evaluated. Well-characterized *in vitro* bioassays for LH have been developed. Rat or mouse Leydig cells are incubated over several hours with various concentrations of LH. The amount of testosterone produced by the Leydig cells is measured. A potential testicular toxin could be studied by exposing it, in various concentrations, with LH to the

Leydig cell preparations and comparing the amount of testosterone produced to that produced by cells exposed to LH alone.

10. The responsiveness of Sertoli cells should be evaluated. Sertoli cell cultures, preferably from postpubertal males, can be used. The production of known secretory products, such as transferrin or androgen-binding protein, following stimulation by FSH, can be measured as an index of their activity. An effect of a toxin would be reflected in that index. A major question is the relationship of *in vitro* toxicity to *in vivo* toxicity, particularly considering the short-term nature of the tests and the long-term nature of *in vivo* exposure. However, some of the *in vitro* tests provide limited opportunity to evaluate human tissue directly with animal models.

11. Competitive mating (heterospermic insemination) should be evaluated as a screening assay. The use of a mixed-insemination assay (91–93) for screening toxicants offers a means of increasing the sensitivity of fertilization assays and should be explored. Rabbits and possibly rats could be used. Semen from exposed and control males would be mixed and inseminated into the same female and the paternity of the offspring established by genetic markers (i.e., eye or coat color). This has good potential for use as a screening assay of superior sensitivity which could simultaneously assess disturbances of sexual behavior, sperm quality, sperm transport in the female, fertilization, and embryonic and fetal development. A limited number of trials with the system should be adequate to determine its utility.

12. The direct assessment of damage to the sperm genome would permit routine screening and monitoring of males for exposure in the workplace to chemicals that may be hazards to their reproductive capacity. Further studies might attempt the following:

(a) Establish the degree of correlation between abnormal sperm head morphology and an aberrant chromosome complement.

(b) Develop sensitive methods for the identification and measurement of alkylated or modified DNA bases.

(c) Improve the methodology to quantitate alkylated amino acids, since there is evidence that alkylation of sperm chromatin proteins also contributes to reproduction failure.

(d) Develop methods to detect damage to sperm chromatin (e.g., enzymatic detection of strand breaks in sperm DNA).

Additional studies could be designed and sponsored to evaluate the suitability of the four techniques discussed above as routine procedures for detection of genetic abnormalities by direct observation of spermatozoa and of the male pronucleus.

REFERENCES

1. Smith, K. D., Rodriguez-Rigau, L. J. and Steinberger, E.: Relation between indices of semen analysis and pregnancy rate in infertile couples. Fertil. Steril. 28: 1314–1319, 1977.
2. Amann, R. P.: A critical review of methods for evaluation of spermatogenesis from seminal characteristics. J. Androl. 2: 39–60, 1981.
3. Barfield, A., Melo, J., Coutinho, E., Alvarezs, F., Faundes, A., Brache, V., Leon, P., Frick, J., Bartsch, G. and Weiske, W. H.: Pregnancies associated with sperm concentrations below 10 million-ml in clinical studies of a potential male contraceptive method, monthly depot medroxyprogesterone acetate and testosterone esters. Contraception 20(2): 121–127, 1979.
4. David, G., Jouannet, P., Martin-Boyee, A., Spira, A. and Schwartz, D.: Sperm counts in fertile and infertile men. Fertil. Steril. 31(4): 453–455, 1979.
5. Zuckerman, Z., Rodriguez-Rigau, L. J., Weiss, D. B., Chowdhury, A. K., Smith, K. D. and Steinberger, E.: Quantitative analysis of the seminiferous epithelium in human testicular biopsies and the relation of spermatogenesis to sperm density. Fertil. Steril. 30: 448–455, 1978.
6. Freund, M.: Standards for the rating of human sperm morphology. Int. J. Fertil. 11: 97–180, 1966.
7. Johnson, L., Petty, C. S. and Neaves, W. B.: A comparative study of daily sperm production and testicular composition in humans and rats. Biol. Reprod. 22: 1233–1243, 1980.
8. Amann, R. P. and Howards, S. S.: Daily spermatozoal production and epididymal spermatozoal reserves of the human male. J. Urol. 124: 211–215, 1980.
9. Smith, K. D. and Steinberger, E.: What is oligospermia? In: The Testis in Normal and Infertile Men, D. Troen and H. Nankin, Eds., Raven Press: New York; pp. 489–503, 1977.
10. Salisbury, G. W., Van Demark, N. L. and Lodge, L. R.: Physiology of reproduction and artificial insemination of cattle, W. H. Freeman and Co.: San Francisco; 788 pp., 1978.
11. MacLeod, J., Hotchkiss, R. S. and Sitterson, B. W.: Recovery of male fertility after sterilization by nuclear radiation. J. Am. Med. Assoc. 187: 637–641, 1964.
12. Amelar, R. D., Dubin, L. and Hotchkiss, R. S.: Restoration of fertility following unilateral orchiectomy and radiation therapy for testicular tumors. J. Urol. 106: 714–718, 1971.
13. Meistrich, M. L., Hunter, N., Suzuki, N., Trostle, P. K. and Withers, H. R.: Gradual regeneration of mouse testicular stem cells after ionizing radiation. Radiat. Res. 74: 349–362, 1978.
14. Rowley, M. J., Leach, D. R., Warner, G. A. and Heller, C. G.: Effect of graded doses of ionizing radiation on human testes. Radiat. Res. 59: 665–678, 1974.

15. Cheviakoff, S., Calamera, J. C., Morgenfield, M. and Mancini, R. E.: Spermatogenesis in patients with lymphoma after treatment with chlorambucil. J. Reprod. Fertil. 33: 155–157, 1973.
16. Sherins, R. J. and DeVita, V. T., Jr.: Effect of drug treatment for lymphoma on male reproductive capacity—studies of men in remission after therapy. Ann. Intern. Med. 79: 216–220, 1973.
17. Sieber, S. M. and Adamson, R. H.: Toxicity of antineoplastic agents in man: chromosomal aberrations, antifertility effects, congenital malformations, and carcinogenic potential. Adv. Cancer Res. 22: 57–155, 1975.
18. Lu, C. C. and Meistrich, M. L.: Cytotoxic effects of chemotherapeutic drugs on mouse testis cells. Cancer Res. 39: 3575–3582, 1979.
19. Torkelson, T. R., Sadele, S. E., Rowe, V. K., Kodama, J. K., Anderson, H. H., Loquvam, G. S. and Hine, C. H.: Toxicologic investigations of 1,2-dibromo-3-chloropropane. Toxicol. Appl. Pharmacol. 3: 545–559, 1961.
20. Whorton, M. D., Krauss, R. M., Marshall, S. and Milby, T. H.: Infertility in male pesticide workers. Lancet 2(8051): 1259–1261, 1977.
21. Glass, R. I., Lyness, R. N., Mengle, D. C., Powell, K. E. and Kahn, E.: Sperm count depression in pesticide applicators exposed to dibromochloropropane. Am. J. Epidemiol. 109(3): 346–351, 1979.
22. Lantz, G. D., Cunningham, G. R., Huckins, C. and Lipshultz, L. I.: Recovery from severe oligospermia after exposure to dibromochloropropane. Fertil. Steril. 35: 46–53, 1981.
23. Amann, R. P.: Use of animal models for detecting specific alterations in reproduction. Appl. Fund. Toxicol.; in press, 1982.
24. Amann, R. P.: Sperm production rates. In: The Testis, Vol. 1, A. Johnson, W. Gomes, and N. Van Demark, Eds., Academic Press, Inc.: New York; pp. 433–482, 1970.
25. Carson, W. S. and Amann, R. P.: The male rabbit. VI. Effects of ejaculation and season on testicular size and function. J. Anim. Sci. 32: 302–309, 1972.
26. Foote, R. H.: Research techniques to study reproductive physiology in the male. In: Techniques and Procedures in Animal Science Research. American Society of Animal Producers: Albany, New York; pp. 81–100, 1969.
27. Robb, G. W., Amann, R. P. and Killian, G. J.: Daily sperm production and epididymal sperm reserves of pubertal and adult rats. J. Reprod. Fertil. 54: 103–107, 1978.
28. Amann, R. P. and Lambiase, J. T.: The male rabbit. III. Determination of daily sperm production by means of testicular homogenates. J. Anim. Sci. 28: 369–374, 1969.
29. Amann, R. P., Kavanaugh, J. F., Griel, L. C., Jr. and Voglmayr, J. K.: Sperm production of Holstein bulls determined from testicular spermatid reserves, after cannulation of rete testis or vas deferens, and by daily ejaculation. J. Dairy Sci. 57: 93–99, 1974.

30. Johnson, L., Petty, C. S. and Neaves, W. B.: A new approach to quantification of spermatogenesis and its application to germinal cell attrition during human spermiogenesis. Biol. Reprod. 25: 217–226, 1981.
31. Clermont, Y.: Kinetics of spermatogenesis in mammals: seminiferous epithelial cycles and spermatogonial renewal. Physiol. Rev. 52: 198–236, 1972.
32. Swierstra, E. E. and Foote, R. H.: Cytology and kinetics of spermatogenesis in the rabbit. J. Reprod. Fertil. 5: 309–322, 1963.
33. Swierstra, E. E., Whitefield, J. W. and Foote, R. H.: Action of amphotericin B (fungizone) on spermatogenesis in the rabbit. J. Reprod. Fertil. 7: 13–19, 1964.
34. Berndtson, W. E.: Methods for quantifying mammalian spermatogenesis: a review. J. Anim. Sci. 44: 819–833, 1977.
35. Lu, C. C., Meistrich, M. L. and Thames, H. D.: Survival of mouse testicular stem cells after gamma or neutron irradiation. Radiat. Res. 81: 402–415, 1980.
36. Amann, R. P.: The male rabbit. IV. Quantitative testicular histology and comparisons between daily sperm production as determined histologically and daily sperm output. Fertil. Steril. 21: 662–672, 1970.
37. Lambiase, J. T., Jr. and Amann, R. P.: The male rabbit. V. Changes in sperm reserves and resorption rate induced by ejaculation and sexual rest. J. Anim. Sci. 28: 542–549, 1969.
38. Fray, C. S., Hoffes, A. P. and Fawcett, D. W.: Reexamination of motility patterns of rat epididymal spermatozoa. Anat. Rec. 173: 301–308, 1972.
39. Gaddum, P.: Sperm maturation in male reproductive tract—development of motility. Anat. Rec. 161: 471–482, 1968.
40. Blandau, R. J. and Rumery, R. E.: Relationship of swimming movements of epididymal spermatozoa to their fertilizing capacity. Fertil. Steril. 15: 571–579, 1964.
41. Lock, L. F. and Soares, E. R.: Increases in morphologically abnormal sperm in rats exposed to ^{60}Co irradiation. Environ. Mutagen. 2: 125–131, 1980.
42. Harasymowyez, J., Ball, L. and Seidel, G. E., Jr.: Evaluation of bovine spermatozoal morphologic features after staining or fixation. Am. J. Vet. Res. 37: 1053–1057, 1976.
43. Holtz, W. and Foote, R. H.: Anatomy of reproductive system in male Dutch rabbits *(Oryctolagus cuniculum)* with special emphasis on the accessory sex glands. J. Morph. 158: 1–20, 1978.
44. Amann, R. P.: Effect of ejaculation frequency and breed on semen characteristics and sperm output of rabbits. J. Reprod. Fertil. 11: 291–293, 1966.
45. Holtz, W. and Foote, R. H.: Sperm production, output, and urinary loss in the rabbit. Proc. Soc. Exp. Biol. Med. 141: 958–962, 1972.
46. Kirton, K. E., Desjardins, C. and Hafs, H. D.: Levels of some normal constituents of rabbit semen during repetitive ejaculation. Fertil. Steril. 17: 204–211, 1966.

47. Paufler, S. K. and Foote, R. H.: Effect of triethylenemelamine (TEM) and cadmium chloride on spermatogenesis in rabbits. J. Reprod. Fertil. 19: 309–319, 1969.

48. Schwartz, D., Laplanche, A., Jouannet, P. and David, G.: Within-subject variability of human semen in regard to sperm count, volume, total number of spermatozoa, and length of abstinence. J. Reprod. Fertil. 57: 391–395, 1979.

49. Katz, D. F. and Overstreet, J. W.: Sperm motility assessment by videomicrography. Fertil. Steril. 35(2): 188–193, 1981.

50. Eliasson, R.: Analyses of semen. In: Progress in Infertility, S. J. Behram and R. W. Kistnes, Eds., Little Brown and Co.: Boston; pp. 691–713, 1975.

51. Holtz, W. and Foote, R. H.: Composition of rabbit semen and the origin of several constituents. Biol. Reprod. 18: 286–292, 1978.

52. Baker, M. W. G., Bremner, W. J., Burger, H. G., de Kretser, D. M., Dulmanis, A., Eddie, L., Hudson, B., Keogh, E. J., Lee, V. W. K. and Rennie, G.: Testicular control of FSH secretion. Rec. Prog. Horm. Res. 32: 429, 1976.

53. de Kretser, D. M., Kerr, J. B., Rick, K. A., Risbridger, G. and Dobas, M.: Hormonal factor involved in normal spermatogenesis. In: Testicular Development, Structure, and Function, A. Steinberger and E. Steinberger, Eds., Raven Press: New York; pp. 109–115, 1980.

54. Psychoyos, A.: Hormonal control of ovoimplantation. Vitam. Horm. 31: 201–256, 1973.

55. Bedford, J. M.: Techniques and criteria used in the study of mammalian fertilization. In: Methods in Mammalian Embryology, J. C. David, Ed., W. H. Freeman and Co.: San Francisco; pp. 37–63, 1971.

56. Yanagimachi, R., Yanagimachi, H. and Rogers, B. J.: Use of zona-free animal ova as a test system for assessment of fertilizing capacity of human spermatozoa. Biol. Reprod. 15(4): 471–476, 1976.

57. Hahn, J., Foote, R. H. and Cranch, E. T.: Tonometer for measuring testicular consistency of bulls to predict semen quality. J. Anim. Sci. 29: 483–489, 1969.

58. Oakberg, E. F.: Degeneration of spermatogonia of the mouse following exposure to X-rays, and stages in the mitotic cycle at which death occurs. J. Morph. 97: 39–54, 1955.

59. Nicander, L.: Studies on the regional histology and cytochemistry of the ductus epididymis in rabbits. Acta Morphol. Neerl. Scand. 1: 99–1[illegible] 1957.

60. Reid, B. L. and Cleland, K. W.: The structure and function of the epididymis. I. The histology of the rat epididymis. Aust. J. Zool. 5: 223–246, 1957.

61. Brooks, D. E.: Biochemical environment of sperm maturation. In: The Spermatozoon: Maturation, Motility, Surface Properties and Comparative Aspects, D. W. Fawcett and J. M. Bedford, Eds., Urban and Schwarzenberg: Baltimore; pp. 23–34, 1979.

62. Brandes, D., Ed.: Male Accessory Sex Organs. Academic Press: New York; 527 pp., 1974.

63. Hammerstedt, R. H.: Characterization of sperm surfaces using physical techniques. In: Spermatozoon: Maturation, Motility, and Surface Properties and Comparative Aspects, D. W. Fawcett and J. M. Bedford, Eds., Urban and Schwarzenberg: Baltimore; pp. 205–216, 1979.

64. Bedford, J. M. and Cooper, G. W.: Membrane fusion events in the fertilization of vertebrate egg. In: Cell Surface Reviews, Vol. 5, Membrane Fusion, Elsevier North Holland: Amsterdam; pp. 66–111, 1978.

65. Mann, T. and Lutwak-Mann, C.: Male Reproductive Function and Semen, Springer-Verlag: New York; 495 pp., 1981.

66. Hammerstedt, R. H.: Monitoring the metabolic rate of germ cells and sperm. In: Reproductive Processes and Contraception, K. W. McKerns, Ed., Plenum Publishing: New York; pp. 353–391, 1981.

67. Hegde, U. C., Shastry, P. R. and Rao, S. S.: Use of dithiothreitol for improved visibility of F-body in human Y-bearing spermatozoa. J. Reprod. Fertil. 53: 403–405, 1978.

68. Evenson, D. P., Darzynkiewicz, Z. and Melamed, M. R.: Relation of mammalian sperm chromation heterogeneity to fertility. Science 210: 1131–1133, 1980.

69. Gledhill, B. L., Lake, S. and Dean, P. N.: Flow cytometry and sorting of sperm and other male germ cells. In: Flow Cytometry and Sorting, M. Melamed, P. Mullaney, and M. Mendelsohn, Eds., J. Wiley and Sons: New York; pp. 471–484, 1979.

70. Rudak, E., Jacobs, P. A. and Yanagimachi, R.: Direct analysis of chromosome constitution of human spermatozoa. Nature 274: 911–913, 1978.

71. Aafjes, J. H., Vels, J. M. and Schenck, E.: Fertility of rats with artificial oligozoospermia. J. Reprod. Fertil. 58(2): 345–352, 1980.

72. Desjardins, C., Kirton, K. T. and Hafs, H. D.: Sperm output of rabbits at various ejaculation frequencies and their use in the design of experiments. J. Reprod. Fertil. 15: 27–32, 1968.

73. Seidel, G. E., Jr. and Foote, R. H.: Variance components of semen criteria from bulls ejaculated frequently and their use in experimental design. J. Dairy Sci. 56: 399–405, 1973.

74. MacLeod, J.: Human male infertility. Obstet. Gynecol. Surv. 26: 335–351, 1971.

75. Janick, J. and MacLeod, J.: Measurement of human spermatozoan mobility. Fertil. Steril. 21: 140–146, 1970.

76. Lubs, H. A.: Testicular size in Kleinfelter's Syndrome in men over fifty. Report of a case with XXY/XY mosaicism. New Engl. J. Med. 267: 326–331, 1962.

77. Asbjornsen, G., Molne, K., Klepp, O. and Aakvaag, A.: Testicular function after combination chemotherapy for Hodgkin's disease. Scand. J. Haematol. 16(1): 66–69, 1976.

78. Van Thiel, D. H., Sherins, R. J., Meyers, G. H. and Devita, V. T., Jr.: Evidence for a specific seminiferous tubular factor affecting follicle-stimulating hormone secretion in man. J. Clin. Invest. 51: 1009–1019, 1972.

79. Cunningham, G. R. and Huckins, C.: Serum FSH, LH, and testosterone in ^{60}Co γ-irradiated male rats. Radiat. Res. 76: 331–338, 1978.
80. Verjans, H. L. and Eik-Nes, K. B.: Hypothalamic-pituitary-testicular system following testicular X-irradiation. Acta Endorcinol. 83(1): 190–200, 1976.
81. MacLeod, J.: Human seminal cytology following the administration of certain antispermatogenic compounds. In: Agents Affecting Fertility, C. R. Austin and J. S. Perry, Eds., Little Brown and Co.: Boston; pp. 93–123, 1965.
82. Belsey, M. A., Eliasson, R., Gallegos, A. J., Maghissi, K. S., Paulsen, C. A. and Prasad, M. R. N.: Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction, Press Concern: Singapore; 43 pp., 1980.
83. National Academy of Sciences: Drinking Water and Health, Vol. 3, National Academy Press: Washington, D.C.; pp. 25–265, 1980.
84. Young, W. C., Ed.: Sex and Internal Secretions, Vol. 1, 3rd Edition, Williams and Wilkins: Baltimore; pp. 161–448, 1961.
85. Young, W. C., Ed.: Sex and Internal Secretions, Vol. 2, 3rd Edition, Williams and Wilkins: Baltimore; pp. 707–796, 1173–1239, 1961.
86. Mann, T.: The Biochemistry of Semen and of the Male Reproductive Tract, Methuen: London; 240 pp., 1964.
87. Hamilton, D. W. and Greep, R. O., Eds.: Handbook of Physiology, Section 7, Vol. 5: Male Reproductive System, American Physiological Society: Washington, D.C.; 519 pp., 1975.
88. Greep, R. O., Koblinsky, M. A. and Jaffee, F. S., Eds.: Reproduction and Human Welfare: A Challenge to Research, MIT Press: Cambridge, Mass.; 622 pp., 1976.
89. Wyrobek, A. J. and Gledhill, B. L.: Human semen assays for workplace monitoring. In: Proceedings of Workshop on Methodology for Assessing Reproductive Hazards in the Workplace. Center for Disease Control, National Institute for Occupational Health and Safety; pp. 327–355, 1980.
90. Gledhill, B. L. Personal communication, Lawrence Livermore Laboratory: California; 1981.
91. Beatty, R. A., Bennett, G. H., Hall, J. G., Hancock, J. L. and Stewart, D. L.: An experiment with heterospermic insemination in cattle. J. Reprod Fertil. 19: 491–502, 1969.
92. Overstreet, J. W. and Adams, C. E.: Mechanisms of selective fertilization in the rabbit: sperm transport and viability. J. Reprod. Fertil. 26: 219–231, 1971.
93. O'Connor, M. T., Amann, R. P. and Saacke, R. G.: Comparisons of computer evaluations of spermatozoal motility with standard laboratory tests and their use for predicting fertility. J. Animal Sci.; in press, 1981.

APPENDIX

DETAILS OF TEST PROTOCOLS AND GLOSSARY OF TERMS FOR MALE RISK ASSESSMENT

I. DESCRIPTION AND DISCUSSION OF TESTS USEFUL IN ANIMAL MODELS OR MAN

Body Weight

Measure body weight of all test animals weekly starting two weeks before administration of the compound and continuing until termination of the study.

Testicular Characteristics

Testis size in situ

The number of spermatozoa, and to a lesser extent the quantity of testosterone, produced by the testes of normal individuals is a function of testis size and, to a lesser extent, of variation in the proportion of the testis composed of germinal elements and interstitial tissue (1). Therefore, assessment of testicular size is very important from a functional standpoint. In scrotal animals, testicular size can be measured easily, accurately, repeatedly and without damage to the individual (2–5). In many species of laboratory and domestic animals, testis size is correlated (correlation coefficient $r = 0.8–0.9$) with sperm output in ejaculated semen when males are ejaculated frequently (e.g., four ejaculates per week) (1, 2). Changes in testis size should be correlated with results of other tests to increase the accuracy of the analysis.

Measurements of testis size should be made biweekly or weekly with animal models and could be made part of an annual physical examination given to men working in a hazardous environment.

Scrotal circumference. This measurement can be taken easily in animals with pendulous scrotum (4). To reduce variation, the measurements should be made with a standard procedure.

Linear measurements. Length and width can be measured in species such as dogs, rabbits, bulls, and horses (1, 2–5). Length and width measurements are correlated with testis weight (≥0.90). If these data are correlated with seminal characteristics, adjustment for time lag in spermatogenesis and sperm transport through the excurrent ducts is necessary.

Testis weight

Each testis must be dissected free from the epididymis and pampiniform plexus, and weighed when model animals are killed or castrated. Testis weight, relative to norms for that breed or strain, can reveal gross differences resulting from a treatment.

Spermatid reserves

Counting of homogenization-resistant spermatid nuclei in testicular homogenates is a simple, accurate, and sensitive method for measuring sperm production. This method can be accomplished with simple equipment and does not require extensive training. The nuclei of elongated spermatids are resistant to mechanical and chemical disruption and are easily identified after physical disruption of testicular tissue (1, 6, 7). With human testes, small biopsies can be used (8), and the tissue should be fixed in glutaraldehyde before homogenization, because some spermatid nuclei may not be fully condensed (9). The interval from when spermatids acquire the resistance to homogenization until spermiation is a constant for a species or strain (1). Thus, the number of resistant spermatids is a direct measure of the production of spermatozoa by the testis and the survival of the precursor spermatogenic cells (1).

Either biopsy material (20 mg or more), a representative sample taken at necropsy, or the entire testes (for rats and rabbits) can homogenized or disrupted ultrasonically (6, 10). Resistant spermatid nuclei are counted in a cytometer (at least 6 chambers per sample). Counts should be expressed on both a per-testis basis and a per-mg-of-parenchyma basis (11). Counts from treated animals should be compared to those for concurrent control males.

The time of this analysis relative to an acute treatment or the onset of chronic treatment can be varied so as to reveal possible damage to cells in specific stages of spermatogenesis. The interval between onset of treatment and evaluation should be expressed in terms of the duration of one "cycle of the seminiferous epithelium" for that species. Preferred times for evaluating agents should be chosen according to the kinetics of spermatogenesis (7, 12–17).

Histopathological analysis of testes

Histologic analyses of testicular biopsies or whole testes must be performed on animal models and, in special cases, could be performed on man. Qualitative and quantitative analyses of increasing complexity yield general or precise information. It is axiomatic that serious disturbance will be evident to the observer using direct simple evaluations of germinal epithelium in histological preparations of whole testes. Threshold effects require a detailed evaluation such as that recommended below. Electron microscopy is not considered to be useful for screening of damaging agents. Testicular tissue must be fixed immediately in Bouin's or Zenker's fluid (10% formalin is *not* satisfactory). Slides should be stained with hematoxylin and eosin for simple analyses and with periodic-acid-Schiff-hematoxylin if a more precise determination of the stages of spermatids is required (17).

Gross morphology. Appearance of Leydig interstitial cells (18); occurrence of lymphoid cell or macrophage infiltration (19); presence of germ cells of each stage (spermatogonia, spermatocytes, spermatids, sperm) in seminiferous tubules (20); presence of large numbers of degenerating (21), multinucleate (22), or abnormal germ cells (23) should be noted.

Nonfunctional tubules. The percentage of tubular cross-sections with *no evidence of spermatogenesis* (i.e., <4 germ cells) should be scored during brief examination at 100X or 400X magnification of 250 cross-sections per testis (24, 25). Such examination could be performed 2 to 7 days after acute treatment or 6 cycles after onset of chronic treatment. The integrity of the layer of Sertoli cells in these sterile tubules should also be noted.

Tubules with spermatids lining the lumen. The end product of spermatogenesis is reflected in the "mature" spermatids about to be released from the Sertoli cells. Tubules with spermatids aligned at the lumen can be easily recognized (16). The incidence of such tubules is a characteristic of the species. Deviations between control and treated males reflect testicular dysfunction.

Seminiferous tubule diameter. Diameters can change with interference of tubular function (26). Measurements of minor diameter should be taken on essentially round tubule cross-sections (cut at right angles to their long axis) taken from several different locations in sections used for scanning other aspects of gross histology. Only tubules in which the minor diameter is within 10% of the major diameter (i.e., the sections are essentially transverse) should be measured.

Counts of preleptotene or leptotene spermatocytes

The number of leptotene spermatocytes per Sertoli cell with a visible nucleolus can be quantitated because of a characteristic nuclear morphology of leptotene spermatocytes (27, 28). T number of leptotene spermatocytes and the number of Sertoli cells with a visible nucleolus should be determined in the same set of tubules. The ratio of spermatocytes per Sertoli cell is a sensitive measure of testicular damage; effects of as little as 5 rad of radiation can be detected (20).

Epididymal Characteristics

Weight of distal half of epididymis

The distal portion of the epididymis can be isolated by severing the corpus epididymidis midway between the caput and cauda and at the junction of the distal cauda with the ductus deferens. The distal epididymis and the contralateral epididymis should be weighed promptly.

Number of sperm in the distal half of epididymis

One epididymis, weighed as above, is homogenized to liberate the spermatozoa contained therein (6, 7); simple mincing of the tissue is inadequate. Sperm cells are counted using a cytometer (at least 6 chambers counted per sample). The results should be expressed as total counts. The epididymis evaluated could be alternated within each control or treated group to ensure representative sampling there is any systematic difference between sides.

Motility of sperm from the distal end

Sperm from the distal end of the remaining cauda epididymidis will be expressed into a phosphate-buffered saline solution containing 5 mM of glucose or pyruvate plus 0.1% bovine serum albumin, polyvinyl alcohol, or similar macromolecules. Sperm concentration should be standardized to 10×10^6 to 40×10^6 per ml, and the percentage of motile sperm determined at 37°C under conditions similar to those described for estimating percentage of motile spermatozoa in ejaculated semen.

Gross morphology of spermatozoa from the distal end

The same semen preparation used for estimating the percentage of motile spermatozoa will be viewed by phase-contrast microscopy at 400X for evaluation of gross morphology. The proportion of the spermatozoa from treated animals, in comparison with controls, with misshapen heads, acrosomal defects or distorted swimming patterns, will be estimated.

Detailed morphology of spermatozoa from the distal end

For detailed evaluation of sperm morphology, smears will be prepared by a procedure minimizing artifacts (29), then stained with eosin-nigrosin (or other differential stain). A total of 200 to 400 spermatozoa should be classified per sample. Smears can be preserved as a permanent record, or videotapes can be prepared. Detailed morphological or morphometric examination is possible with either.

Accessory Sex Gland Characteristics

1. The accessory sex glands are biomonitors of androgen production by the testes. Thus, accessory sex gland weight will be recorded when each male animal is killed.
2. For rats, the vesicular glands are discrete organs and very easily distinguishable. After removal and expression of the viscid fluid, the glands should be blotted and weighed.
3. The individual accessory sex glands are not discrete in the rabbit (30). Thus, the total set of accessory glands will be excised as a single unit, blotted, and weighed. The organs will be reweighed after removal of any secretion present in the vesicular glands.

Seminal Analysis

General aspects of seminal analysis

(a) Analysis of semen offers a convenient approach for monitoring function of the germinal epithelium and, with less specificity, the functions of the epididymides, prostate, vesicular glands, and bulbourethral glands (11). An abnormality in epididymal function may be detected in semen ejaculated 3 to 15 days after epididymal dysfunction. An abnormality in spermatogenesis typically cannot be detected in semen until after at least 1 to 4 cycles of the seminiferous epithelium (16–64 days in man or 11–43 days in rabbit) have passed (1, 11, 14, 31), plus time for epididymal transport. This long interval is required because an agent must accumulate to a toxic concentration and produce a lesion in germ cells at a specific point in their development (often >2 cycles of the seminiferous epithelium before the end of spermatogenesis) before production of more mature germ cells is affected. After passage of a given interval, evidence of the lesion can be seen within the testis but the lesion will not be evident in semen until the affected germ cells have completed spermatogenesis (2–3 cycles), passed through the epididymis (4–16 days), and appeared as spermatozoa in ejaculated semen.

(b) Multiple samples can and should be obtained from each individual male. Both quantitative and qualitative characteristics of more than one ejaculate must be evaluated to gain a reasonable understanding of testicular function (1, 11, 32). Data for samples collected before experimental exposure can be used as one basis for assigning males to control or treatment groups or as a covariant in the statistical analysis.

(c) The species, strain, and age of males; testicular size; season; method of semen collection; and interval since the previous ejaculation(s) all influence quantitative characteristics of semen and must be carefully controlled (11, 33).

(d) If seminal analyses are planned, use of a species from which semen can be collected by artificial vagina or digital manipulation (masturbation) is essential. Suitable species include man, rabbit, dog, bull, and minipig (1, 2, 11, 32–34). The rabbit is the species of choice for screening potentially toxic agents because of size, availability, cost, and ease of use. Small rabbits (e.g., Dutch Belted) are as good as larger breeds (e.g., New Zealand White) and are cheaper to house. Rams, goats, and stallions are less ideal because seasonal changes are more profound. Subhuman primates probabl will not be used frequently because of their limited numbers and cost. Although useful in many other aspects, rats have limited use (as do mice) because of their small seminal volume, difficulty in quantifying seminal characteristics, and the necessity to use electrostimulation for semen collection. Improved procedures for quantitative collection of semen from rats or mice are unlikely to overcome their limitations. However, as outlined above, cauda epididymal sperm can be obtained (on a one-time basis) from a mouse or rat and evaluation of the motility and morphology of epididymal sperm is desirable.

(e) For most studies, sexually mature males (body weight ≤90% of maximum value for that strain) should be used. If consequences of exposure before puberty are to be evaluated, the age or body weight when a given number of sperm are first ejaculated may be a useful criterion (requires at least weekly testing) and the postpubertal changes in semen quality could be monitored (33).

By monitoring seminal characteristics longitudinally from exposure, through a reasonable interval when an effect might be expressed (6 X the duration of the cycle of the seminiferous epithelium for the species studied) and during a recovery phase (if appropriate) of twice this duration, information can be obtained on the point when damage is expressed and when recovery occurs (1, 2, 14).

Volume

(a) Volume of the ejaculate should be measured with an accuracy of greater than 90 to 95%. To measure accurately ejaculates with a small volume, it is recommended that collection tubes be preweighed and ejaculate volume be calculated from the weight, assuming a specific gravity of 1.0.

(b) Systematic errors associated with seminal loss during collection or transfer to a measuring device should be minimized. Measurement of ejaculate volume within the collection vessel, after addition of a known volume of buffer if essential, is desirable. Systematic errors in measurement often can be corrected for (1, 2, 11).

(c) If a uniform collection interval and standardized collection procedure are used (1, 11), differences of >25% in ejaculate volume probably could be detected in a longitudinal study utilizing ten rabbits per treatment group (35). A difference of this magnitude probably would reflect abnormal function of the accessory sex glands if unaccompanied by a change in sperm output.

(d) The coefficient of variation for volume of a human ejaculate is unknown but could be calculated from available data. It is likely that a sizable number of ejaculates must be evaluated for each individual in a group to detect a 25% change in seminal volume.

Seminal plasma constituents

(a) The biochemical components of seminal plasma may reflect the functionality of the epididymides and accessory sex glands (11), but the concentration of a compound in a seminal plasma has limited diagnostic value (11).

(b) If collection procedures are rigidly standardized for a species, a marked change (>25%) in the total mass of a constituent ejaculated, could reflect the function of the excurrent duct system or one or more accessory sex glands. It is not clear at present, however, whether any constituents change their concentration markedly in the course of repeated ejaculations with humans (see Research Needed).

Spermatozoal concentration

(a) The term spermatozoal *concentration* is preferred to those of sperm count or sperm density.

(b) Sperm concentration, by itself, provides little information (11), but sperm concentration must be determined accurately so that the total number of sperm per ejaculate can be calculated (see below).

(c) Sperm concentration should be determined using a calibrated spectrophotometer or electronic cell counter, if contaminating cells or debris are not a problem, because of their accuracy and precision (2, 11). If extraneous material is present in the semen, visual counts using a cytometer are essential. Use of a cytometer (with a phase-contrast microscope) is time consuming, and ⩾6 replicate counts are necessary to achieve ⩾90% accuracy for a single sample.

Total sperm per ejaculate

(a) The term total number of sperm per ejaculate (volume × sperm concentration) is preferable to that of total sperm count.

(b) Total sperm per ejaculate represents the number of sperm coming from the excurrent duct system and is independent of the degree of dilution by accessory sex gland fluid (11).

(c) When semen is collected by a uniform procedure and total sperm per ejaculate is averaged over time, daily sperm output (number of sperm in a series of ejaculates divided by the time span) can be calculated. Daily sperm output, in rabbits and bulls, is highly correlated (≅0.9) with daily sperm production (1, 11, 36).

(d) To measure daily sperm output accurately (1, 11), a uniform interval of one, two, or three days between semen collections is essential, and the series of ejaculates should extend over 14 (preferably 20) days (data for the first 3–6 ejaculates should be excluded a priori and data for the remaining >6 samples averaged).

(e) If semen is collected infrequently (one ejaculate weekly), a 50% reduction in sperm production probably would be undetectable (11). To have a 75% chance of detecting a difference of 50% daily sperm output would require about 20 rabbits per treatment and ejaculation for >5 weeks (35).

(f) A formula relating the coefficient of variation (CV) between counts; the significance level at which the statistical test is to be performed, α (e.g., $\alpha = 0.05$, $\alpha = 0.01$); the desired power or sensitivity of the statistical test, $1\text{-}\beta$ (e.g., $1\text{-}\beta = 0.50$, $1\text{-}\beta = 0.90$); and the sample size of the study, N (i.e., N treated and N control animals), to the required change (in terms of percent of 'normal' or control values) in the test criteria is given by

$$\frac{(Z_\alpha + Z_{1\text{-}\beta})(CV)}{\sqrt{N/2}} = \%\ \text{change}$$

where $Z_\alpha = 1.645$ for $\alpha = 0.05$ and $Z_\alpha = 2.326$ for $\alpha = 0.01$, and $Z_{1\text{-}\beta} = 0, 0.253, 0.524, 0.842$, and 1.281 for $1\text{-}\beta = 0.5, 0.6, 0.7, 0.8$, and 0.9, respectively. It should be noted that this formula assumes a one-sided statistical test, that is, looking for changes between treated and control animals in only one direction (e.g., decrease in sperm concentration). Along with the coefficients of variation given in Table 10, it can be used to determine the adequacy of different experimental designs. For example, the largest coefficient of variation, other than for accessory sex gland weight, is for the test criterion total sperm/ejaculate in Dutch Belted rabbits, $CV = 0.75$. Assuming that any statistical comparison between 12 treated and 12 control rabbits is conducted at the $\alpha = 0.05$ level (i.e., 5% test level), to have at least a 50% chance of detecting a statistically significant difference (i.e., power = 0.50), then the treatment must produce at least a 50% change in the test criterion, that is, $(1.654 + 0)(0.75)/\sqrt{12/2} = 0.5$. Because each of the other criteria, except for accessory sex glands, have coefficients of variation of less than 0.75, they would have the same power, 50%, of detecting a smaller effect; for example, for testis weight, $CV = 0.2$ giving a percent change of 13%, $(1.645 + 0)\ (0.20)/\sqrt{12/2} = 0.13$.

Sperm motility

(a) Rigid control of temperature at 37°C and other conditions of evaluation are essential (2, 11).

(b) Visual evaluation of sperm motility using diluted semen and a phase-contrast microscope is informative and rapid, although subjective. Visual estimations are adequate for an initial screen, provided that control and treatment samples are presented randomly in a blind manner to the observer. For second- or third-level analysis, more objective procedures, such as videotape and analyses or track motility (37–39) should be considered.

(c) The percentage of progressively motile sperm, the translatory velocity, and the presence of sperm moving in a circular pattern or backward should be recorded.

(d) A reduced percentage of motile sperm might reflect abnormal spermatogenesis, abnormal functions of the epididymis or entrance of an antimotility factor into the semen (via the excurrent ducts, prostate, bulbourethral glands, or vesicular glands) where it could exert a direct effect on the sperm.

(e) The percentage of motile sperm probably reflects both normality of spermatogenesis and sperm metabolism.

(f) Variation within males in the percentage of motile spermatozoa (and probably velocity) is less than for ejaculate volume or total sperm per ejaculate (35, 40).

(g) A significant decrease in the percentage of motile sperm would be a strong indicator for a potential decline in fertility and especially so if sperm numbers are limited.

Spermatozoal morphology

(a) Abnormalities of sperm morphology reflect dysfunction of the germinal epithelium (primary abnormality) or of the excurrent duct system (secondary abnormality). Certain abnormalities cannot be clearly attributed to a specific site of action.

(b) An increase in the percentage of abnormal sperm may precede a decline in the total number of sperm per ejaculate (if any) and can serve as a sensitive indicator of epididymal or testicular function.

(c) Within a male, sperm morphology is quite consistent over time (41). This consistency makes sperm morphology a sensitive probe while requiring fewer samples per male for an experiment of a given precision.

(d) Evaluation of sperm morphology is subjective (42) and must be carefully standardized among laboratories (11, 42, 43). A detailed classification probably is unnecessary in an initial screening process. Classification of spermatozoa, based on light microscopy, as normal or abnormal head, normal or abnormal tail is adequate for a screen. For second- or third-level screening, a more complex classification might be used (42–44).

(e) Evaluation of sperm morphology using wet preparations and phase-contrast microscopy is recommended for simplicity and freedom from artifacts (11, 29), although preparation and retention of stained smears (a simple stain like eosion-nigrosin or eosin-analine blue) is desirable for archival purposes.

Ejaculated sperm as an *in vitro* test system

(a) Substances can pass from blood into semen via the fluid from the excurrent ducts and accessory sex glands and could be spermicidal or alter sperm function.

(b) Agents can be screened economically by incubating sperm *in vitro* under standard conditions in a protein-containing buffer at 37°C for 4 to 8 hours. Sperm could be exposed to the agent briefly (10–30 minutes) or throughout the incubation period. A dose-response curve should be established using objective methods of evaluation and sperm from humans or other species (rats, rabbits, dogs, or bulls).

(c) The decline in percentage of motile sperm over time is an excellent criterion, but other criteria (e.g., integrity of the acrosome and plasma membrane, oxygen consumption, adenosine 5′-triphosphate content, or degree of agglutination [45]) could be used.

(d) Compounds that are spermicidal *in vitro* at concentrations that could be anticipated or shown to be present in blood or seminal plasma should be carefully screened *in vivo*. Failure to demonstrate a spermicidal action *in vitro* is *not* evidence that an agent would be free of effects on male reproduction, nor is spermicidal action *in vitro* evidence of *in vivo* activity.

Assessment of Male Reproductive Toxicity Using Endocrinological Methods

General

(a) This section describes general aspects of applying endocrinological methods to the study of male reproduction. Specific applications of these techniques to studies in animals and men are described elsewhere in this account.

Normal male reproductive function requires hormonal stimulation of the testes and production by the testes of adequate numbers of sperm and the hormone testosterone. Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are the two hormones necessary to maintain normal testicular function. These hormones originate in the pituitary gland and travel through the blood to affect the testis. The production of LH and FSH and their release from the pituitary are stimulated by gonadotropin-releasing hormone (GnRH), which is produced in the hypothalamus at the base of the brain. Testosterone and other hormones produced by the testis are carried by the blood throughout the body. At the pituitary, these hormones tend to decrease the production of LH and FSH, that is, they exert a "negative feedback" effect on LH and FSH secretion.

(b) If a defect occurs in hypothalamic or pituitary function, blood levels of FSH and LH will tend to decrease.

(c) If a defect occurs in the testis (either in sperm or testosterone production), FSH and LH levels will tend to *increase* because of lack of the "negative feedback" effect of testicular hormones.

(d) In addition to its effects on the pituitary, testosterone exerts many effects throughout the body. It is necessary for expression of male sexual behavior and the ability to perform intercourse, stimulates muscle and bone development and red blood cell production, and is essential for many other aspects of normal body function. A decrease in blood levels of testosterone can be expected to affect all these functions adversely.

(e) It is clearly established in all mammalian species investigated that an endocrine defect in the brain, pituitary, or testis may inhibit spermatogenesis and normal sexual behavior and cause sterility. Less severe defects in these tissues (not so severe as to lead to infertility) might be detected by measurements of hormone concentration in the blood. In certain situations, including studies of human beings, hormone measurements are very practical, because they can be performed on ordinary samples of blood serum, whereas seminal fluid or testicular tissue may be difficult or impossible to obtain.

(f) Hormonal measurements are important and sensitive tools in the assessment of toxicity to the male reproductive system. They can be compared directly among a variety of species and between control and treated groups of any species including man. Hormonal data may give a clue as to the tissue in which a toxic effect is occurring.

Hormone assay and application

(a) Hormones commonly are measured by radioimmunoassay. This is an extremely sensitive technique and, when done in a competent laboratory, is quite reliable for measuring testosterone, FSH, and LH. Many commercial laboratories perform radioimmunoassays for LH, FSH, and testosterone in human blood samples.

Developing these assays requires familiarity with the technique, access to counters for radioactivity and availability of specialized assay reagents such as purified preparations of the hormones and antibodies to these hormones.

(b) Testosterone is an identical molecule in all species, so it can be measured from any species in a single assay. Nevertheless, appropriate species controls are essential to preclude the possibility that a cross-reacting molecule is altering test results. Both LH and FSH are protein hormones that differ slightly in molecular structure among species. Assays for LH and for FSH tend to be species-specific, so that assay reagents appropriate for the species in question generally are required for measuring LH or FSH. For rats and man these reagents are widely available, and such reagents have recently been introduced for rabbits. For normal adult male rats, however, the concentration of LH in peripheral blood is usually below the sensitivity level of available radioimmunoassays.

Measurement of blood levels of hormones yields a direct assessment of the level of exposure necessary to produce a toxic effect. The methodology is sufficiently accurate to detect changes of 20% in mean hormone levels using generally available numbers of animals (e.g., 20 per group). Statistical adequacy for these assays when performed competently is very good; in general, within-assay coefficients of variations are below 10%, and between-assay coefficients of variations are less than 15% (46). These assays are specific measures of reproductive toxicity; disease of other organ systems will not affect these measurements unless the disease concomitantly affects the reproductive system.

Any statistically significant difference between hormone levels in a comparison of control and exposed animals or men can be accepted as strong evidence for a toxic effect of the exposure on reproduction. If such an effect were established by animal studies, this could be used as strong evidence that a similar effect would occur in human beings. In essentially every instance studied in detail, agents found to be testicular toxins in one species have a similar effect in other species (47).

(c) Hormone levels may be measured in single blood samples, although evaluation of several samples taken at two-hour intervals is better. The time of day should be standardized because of diurnal rhythms. Ordinarily, no special preparation is necessary concerning diet or physical activity.

(d) For all known reproductive toxins, the damage to reproduction is reversible if the exposure level is so low as to produce only a minimally detectable effect. With a more severe insult or a prolonged toxin exposure, the damage may be irreversible.

Examination of Known Toxic Exposures

Humans

Seminal fluid analyses. At least one and preferably five seminal fluids obtained by masturbation at two-day intervals should be submitted by each man. Precautions must be taken to ensure ar accurate measurement of seminal volume (11). The age of the individuals and the abstinence interval between samples should also be considered in the evaluation (48–50). Variability among ejaculates from the same individual is influenced by the length of abstinence (50). The semen should be analyzed for volume, sperm concentration, and total sperm per ejaculate. Total sperm per ejaculate must be calculated and compared with norms. Sperm motility (at 37°C) should be objectively assessed using phase-contrast microscopy; motility should be characterized in terms of percentage of motility and velocity. If feasible, videotapes should be made of the living sperm cells, with the tapes being subsequently analyzed in a laboratory familiar with this technique. Seminal smears should be fixed (29) for subsequent analysis. Tests of sperm function such as penetration of zona-free hamster eggs may not be feasible for field work. However, if persistent infertility remains undiagnosed after completion of the other studies proposed, the hamster egg *in vitro* penetration test (51) should be considered.

Blood hormone levels. Peripheral venous blood samples should be obtained at a standardized time of day (preferably 0700–0900 hours) for measurement of serum luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone by radioimmunoassay. Hormone measurements on both the exposed and control groups should be performed in the same laboratory. This laboratory must be one that is recognized for reliability and that maintains carefu quality control records.

Gonadotropin-releasing hormone (GnRH) test. This test has been demonstrated to be capable of detecting mild degrees of primary testicular dysfunction insufficient to elevate basal hormone levels out of the normal range (52). It is not necessary if the basal, unstimulated-hormone levels are abnormal. Blood samples for measurement of LH and FSH are obtained before and at 30, 60, and 90 minutes after administration of GnRH (100 μg i.v.). Synthetic GnRH is now available for use as an investigational new drug through several pharmaceutical companies and will probably be approved by the Food and Drug Administration for general use.

Animal models

Rats. Twenty mature male rats of a highly fertile strain (body weight must be >90% of adult normal for that strain) will be given ⩾0.5 of the maximum tolerated dose (MTD) by inhalation, intraperitoneal (i.p.) injection, drinking water, or gavage for a period of exactly 6 cycles of seminiferous epithelium (12.9 days per cycle X 6 = 77 days). An appropriate control group (or groups) will be evaluated concurrently. If the agent is given by injection or gavage, both nonhandled and vehicle-injected control groups are necessary. Each rat will be weighed weekly starting 14 days prior to initial dosing (day −14) and continuing to day 78. Each male will be caged with two sexually mature, virgin female rats between days 65 and 71 (for a total of 6 nights) to evaluate fertility. On day 78, blood will be taken by cardiac puncture immediately (<1 minute) after removing a male rat from his cage in the animal room and the male rat killed. Serum will be frozen.

Both testes will be weighed. One will be fixed in Bouin's fluid for histologic examination and determination of the number of leptotene spermatocytes per Sertoli cell nucleolus. The second testis will be homogenized and the number of resistant spermatid nuclei determined. The vesicular glands will be weighed as an indirect measure of circulating testosterone concentration. The distal half of one epididymis (half corpus plus cauda) will be weighed. Spermatozoa will be expressed from the severed end of the distal cauda epididymidis into phosphate-buffered saline containing 0.1% bovine serum albumin. The percentage of progressively motile spermatozoa will be determined under phase-contrast microscopy and the incidence of abnormal spermatozoa recorded. A stained slide of the spermatozoa will be made for documentation. The distal half of the contralateral epididymis will be isolated, homogenized, and the total number of sperm heads determined. Concentration of testosterone, LH, and FSH in serum will be determined.

The females will be killed on day 83 to 89 (18 days after mating), and the numbers of corpora lutea and implantation sites, as well as embryo viability, will be determined.

Rabbits. Twelve mature male rabbits (body weight must be ⩾90% of adult normal for that strain) will be given the ⩾0.5 MTD by inhalation, i.p. injection, or in drinking water (the same route of administration should be used for both rats and rabbits when possible) for a period of 6 cycles of seminiferous epithelium (10.7 days per cycle X 6 = 64 days). An appropriate control group (or groups) will be established concurrently. Testis size and body weight will be measured weekly starting on day –14 and continuing through day 65. Two ejaculates will be collected every 3 to 4 days (e.g., Monday and Thursday) using an artificial vagina. The volume, with and without gel, concentration, and total sperm per ejaculate will be measured. Sperm motility and gross sperm morphology will be determined using phase-contrast microscopy. Libido will be subjectively assessed weekly. Each male will be mated with two virgins sexually mature females over a 4-day period (between days 54 and 57) and the females allowed to kindle. On day 65, blood will be taken by cardiac puncture and the male rabbits killed. Evaluation will be similar to that for rats. Testis size and weight, number of homogenization resistant spermatids, weight and sperm content of the distal epididymis, and motility and morphology of sperm from the distal epididymis will be evaluated. Gross histologic evaluation and enumeration of the number of leptotene spermatocytes per Sertoli cell nucleolus will be made on one testis fixed in Bouin's fluid. Weight of the accessory sex glands will be recorded as an indirect measure of circulating-testosterone level. Blood will be saved for possible assay for FSH, LH, and testosterone.

Pregnancy rate and litter size will be determined for females bred to each male.

Fertility Testing

Tests available

Humans. *In vitro* oocyte penetration tests are the only means available for assessing the fertilizing capacity of human sperm. Since human *in vitro* fertilization cannot itself be used as a test, substitutes must be used for the human ovum. These include the zona pellucida of stored human follicular oocytes (53) and the zona-free hamste vitellus (54). Regrettably, these tests have not been carefully validated to establish variation among independent analyses of the same ejaculate or of different ejaculates from one male. In situations where *in vitro* testing of human sperm fertility is indicated, the use of a double-fluorescent-label competitive sperm penetration assay with the zona-free hamster egg will increase the sensitivity of the test (55). The sensitivity of the hamster egg penetration assay is also increased by attempting to count the total number of sperm per penetrated hamster vitellus as well as the percentage of penetrated eggs.

Animals. *In vivo* mating tests should be carried out with laboratory rats and rabbits. A visible reduction of quality of the ejaculate may not be reflected in the fertility level because of a superfluidity of spermatozoa in the ejaculate. The sensitivity of the test can be increased greatly by insemination with critical numbers of sperm (ca. 1×10^6 to 2×10^6 in the case of the rabbit). *In vitro* tests are unnecessary for use with animals.

Usefulness

The animal tests are useful since they measure the ability of sperm to reach and fertilize an ovum. The *in vivo* tests are well established and reliable if critical sperm numbers are used. Tests of the fertilizing capacity of human sperm allow assessment of semen from a human population at reproductive risk when the hazard being studied has produced fertilization dysfunction in the animal tests. If fully validated, the *in vitro* human fertilization system also could be used to determine the dose-response relationship of a compound to the fertility of human sperm.

Sensitivity

Although simple mating trials with evaluation of offspring provide some useful information on fertilization, this is an all-or-none measurement. Since sperm production is greatly in excess of that required for fertility, significant reductions in sperm output by the testes may not be detectable by this method (2). The test can be improved as an assay of fertilization ability by using artificial insemination with sperm in limited numbers. Another potentially useful approach involves competition between two populations of spermatozoa from males whose status in relation to each other is known, with expectation of change in the competitive relations following exposure of the male to an agent of interest (39). This latter approach requires further validation (see Research Needed).

In fertility tests embryos should be recovered as early as the 2–8 cell stage. Evaluation of a second group of pregnant rats between days 15 and 19 enables comparison of the numbers of viable and dead embryos with the number of corpora lutea and is more efficient than allowing parturition. If rabbit eggs of 2–8 cells are recovered, the number of sperm associated with fertilized and unfertilized eggs can be counted. This should bring to light abnormalities in sperm transport or abnormalities of cleavage that may result from defects in the sperm genome (see Sperm Nucleus Integrity).

Specificity

The fertilization tests are specific indicators of reproductive toxicity, although they have limited value in the broader context of toxicological testings. The toxicological end points in the *in vivo* animal fertilization system include failures of (a) sperm-egg association, (b) sperm penetration of the zona pellucida, and (c) normal cleavage of the early embryo. All of these events are directly analogous to those occurring in humans. A consistent failure of human spermatozoa to penetrate >10% of zona-free hamster eggs probably reflects an abnormality of the physiological events associated with fertilization (i.e., sperm capacitation and/or the acrosome reaction). These events are presumed to be the same as those required for human fertilization *in vivo*.

Sperm Nucleus Integrity

A toxic chemical may cause infertility of exposed males through action on the sperm genome rather than by alteration of the normal course of spermatogenesis. Thus, the usual parameters used to assess semen quality will not detect this cause of infertility. Genetic damage to the spermatozoa is best assessed by mating the exposed male to untreated females and observing the progeny for sterility, heritable translocations, sex-chromosome loss, specific locus mutations, mutations affecting the skeleton and eye, and dominant lethality. These procedures are covered in the U.S. Environmental Protection Agency's proposed Guidelines for Mutagenicity Risk Assessment (56). Of these tests, only dominant lethality has a bearing on the fertility of an exposed male. This effect can be detected by evaluation of fertilized eggs and embryos as outlined in the section titled Fertility Testing.

Since the animal tests referred to above are not applicable to the human male, it would be desirable to be able to assess the genetic integrity of human spermatozoa directly. Four methods for the detection of chromosomal abnormalities in spermatozoa are as follows:

Quinacrine staining for Y-chromosome aneuploidy

This technique, used also by inference for possible somatic chromosome aneuploidy (57), is easy and economical, does not require sophisticated equipment, and should be suitable for the study of population groups. However, the method is subject to many errors, and its accuracy has been questioned (58, 59). The reliability of the procedure can be improved by using more rigid criteria for scoring fluorescent Y bodies (60). Nevertheless, the technique's reliability has not been sufficiently established to warrant its use as a routine screening procedure.

Spermatozoal morphology

Chemicals, radiation, heat, and a variety of insults increase the proportion of morphologically abnormal sperm in the ejaculate, and an increase in the number of abnormal sperm usually results in impaired fertility. This aspect has been considered in the section on evaluation of semen quality. That these abnormalities are associated with chromosomal damage, however, has not been demonstrated.

Karyotyping of human spermatozoa by the denuded-hamster-egg technique

This procedure is technically difficult, requires highly trained personnel, and at present should be reserved for evaluation of those cases where additional evidence that chromosome abnormalities are a factor in reduced fertility is desired.

Genetic damage with consequences for male fertility also can arise from strand breaks, base alterations, and base substitution in the sperm DNA. Except for strand breaks, methods are currently unavailable for the detection of these lesions. Future work to develop qualitative and quantitative procedures for the detection of such lesions in sperm DNA would be desirable, since sperm with an apparently normal chromosome complement may be responsible for male fertility problems.

Dose Response

1. The criteria for evaluating male reproductive processes, discussed above, can be quantified. In most cases, the procedures are objective, accurate, precise, and sensitive. Data for each criterion have a considerable response span, although values for normal individuals may not have a normal distribution.

2. A number of agents acting on the reproductive system are known to induce a partial suppression in one or more of the criteria listed when given in low dosages and a more severe effect as the dose is increased.

3. It is likely that separate dose-response curves can be established for several criteria with each agent tested. The sensitivity of a particular test would depend upon the nature of the agent.

4. It is likely that agents affecting reproduction have a threshold dose below which damage does not occur.

5. It is unlikely, however, that chronic administration of an agent at ⩾0.5 MTD would not induce a detectable alteration in one or more of the criteria listed, in at least one of two species, if the agent in fact has a deleterious effect on reproductive function in the human male.

6. Reversibility of damage to the male reproductive system often occurs after exposure to the causative agent is terminated. Complete regeneration repair usually will require an interval equivalent to at least three to four and often more than six to twelve cycles of the seminiferous epithelium.

REFERENCES

1. Amann, R. P.: Sperm production rates. In: The Testis, Vol. 1, A. Johnson, W. Gomes, and N. Van Demark, Eds., Academic Press, Inc.: New York; pp. 433–482, 1970.
2. Foote, R. H.: Research techniques to study reproductive physiology in the male. In: Techniques and Procedures in Animal Science Research, American Society of Animal Producers: Albany, New York; pp. 81–100, 1969.
3. Carson, W. S. and Amann, R. P.: The male rabbit. VI. Effects of ejaculation and season on testicular size and function. J. Anim. Sci. 34:302–309, 1973.
4. Coulter, G. H. and Foote, R. H.: Bovine testicular measurements as indicators of reproductive performance and their relationship to productive traits in cattle: a review. Theriogenol. 11:297–311, 1979.
5. Paufler, S. K., Van Vleck, L. D. and Foote, R. H.: Estimation of testicular size in live rabbits. Int. J. Fertil. 14:188–191, 1969.
6. Amann, R. P. and Lambiase, J. T.: The male rabbit. III. Determination of daily sperm production by means of testicular homogenates. J. Anim. 28:369–374, 1969.
7. Robb, G. W., Amann, R. P. and Killian, G. J.: Daily sperm production and epididymal sperm reserves of pubertal and adult rats. J. Reprod. Fertil. 54: 103–107, 1978.
8. Johnson, L., Petty, C. S. and Neaves, W. B.: The relationship of biopsy evaluations and testicular measurements to overall daily sperm production in human testes. Fertil. Steril. 34: 36–40, 1980.
9. Johnson, L., Petty, C. S. and Neaves, W. B.: A new approach to quantification of spermatogenesis and its application to germinal cell attrition during human spermiogenesis. Biol. Reprod. 25: 217–226, 1981.

10. Mian, T. A., Suzuki, N., Glenn, H. J., Haynie, T. P. and Meistrich, M. L.: Radiation damage to mouse testis cells from ^{99}M technetium pertechnetate. J. Nucl. Med. 18: 1116–1122, 1977.

11. Amann, R. P.: A critical review of methods for evaluation of spermatogenesis from seminal characteristics. J. Androl.; in press, 1981.

12. Lu, C. C., Meistrich, M. L. and Thames, H. O., Jr.: Survival of mouse testicular stem cells after gamma or neutron irradiation. Radiat. Res. 81: 402–415, 1980.

13. Meistrich, M. L., Hunter, N., Suzuki, N., Trostle, P. K. and Withers, H. R.: Gradual regeneration of mouse testicular stem cells after ionizing radiation. Radiat. Res. 74: 349–362, 1978.

14. Paufler, S. K. and Foote, R. H.: Effect of triethylenemelamine (TEM) and cadmium chloride on spermatogenesis in rabbits. J. Reprod. Fertil. 19: 309–319, 1969.

15. Swierstra, E. E. and Foote, R. H.: Cytology and kinetics of spermatogenesis in the rabbit. J. Reprod. Fertil. 5: 309–322, 1963.

16. Clermont, Y.: Kinetics of spermatogenesis in mammals: seminiferous epithelial cycles and spermatogonial renewal. Physiol. Rev. 52: 198–236, 1972.

17. Berndtson, W. E.: Methods for quantifying mammalian spermatogenesis: a review. J. Anim. Sci. 44: 818–833, 1977.

18. Meistrich, M. L., Reid, B. O. and Barcellona, W. J.: Changes in sperm nuclei during spermiogenesis and epididymal maturation. Exp. Cell. Res. 99: 72–78, 1976.

19. Jackson, H.: Comparative effects of some antispermatogenic chemicals. In: The Regulation of Mammalian Reproduction, S. Segel, R. Crozier, P. A. Corfman, and P. D. Condliffe, Eds., Charles C. Thomas: Springfield, Illinois; pp. 257–270, 1973.

20. Oakberg, C. F.: Gamma-ray sensitivity of spermatogonia of the mouse. J. Exp. Zool. 134: 343–356, 1957.

21. Parvinen, L. M., Soderstrom, K. O. and Parvinen, M.: Early effects of vinblastine and vincristine on the rat spermatogenesis: analyses by a new transillumination-phase contrast microscopic method. Exp. Pathol. 15(2): 85–96, 1978.

22. Homm, R. E., Rustians, C. and Hahn, D. W.: Antispermatogenic effects of 5-Thio-D-glucose in male rats. Biol. Reprod. 17(5): 697–700, 1977.

23. Steinberger, E. and Sud, B. N.: Specific effect of fluoroacetamide on spermiogenesis. Biol. Reprod. 2: 369–375, 1970.

24. Lu, C. C. and Meistrich, M. L.: Cytotoxic effects of chemotherapeutic drugs on mouse testis cells. Cancer Res. 39: 3575–3582, 1979.

25. Withers, H. R., Hunter, N. M., Barkley, H. T. and Reid, B. O.: Radiation survival and regeneration characteristics of spermatogenic stem cells of mouse testis. Radiat. Res. 57(1): 88–103, 1974.

26. Courot, M.: Some results obtained in the irradiation with X-rays of testes of lambs. In: Effects of Ionizing Radiation on the Reproductive System, W. Carlson and F. Gassner, Eds., Pergammon Press: New York: pp. 279–286, 1964.

27. Berndtson, W. E.: Methods for quantifying mammalian spermatogenesis: a review. J. Anim. Sci. 44: 818–833, 1977.

28. Steinberger, E. and Tjioe, D. Y.: A method for quantitative analysis of human seminiferous epithelium. Fertil. Steril. 19: 960–970, 1968.

29. Harasymowycz, J., Ball, L. and Seidel, G. E., Jr.: Evaluation of bovine spermatozoal morphologic features after staining or fixation. Am. J. Vet. Res. 37: 1053–1057, 1976.

30. Holtz, W. and Foote, R. H.: Composition of rabbit semen and the origin of several constituents. Biol. Reprod. 18: 286–292, 1978.

31. Amann, R. P.: Use of animal models for detecting specific alterations in reproduction. Appl. Fund. Toxicol.; in press, 1982.

32. Boucher, J. H., Foote, R. H. and Kirk, R. W.: The evaluation of semen quality in the dog and the effects of frequency of ejaculation upon semen quality, libido, and depletion of sperm reserves. Cornell Vet. 48(1): 67–86, 1958.

33. Amann, R. P.: Effect of ejaculation frequency and breed on semen characteristics and sperm output of rabbits. J. Reprod. Fertil. 11: 291–293, 1966.

34. Freund, M.: Effect of frequency of emission on semen output and an estimate of daily sperm production in man. J. Reprod. Fertil. 6: 269–286, 1963.

35. Desjardins, C., Kirton, K. T. and Hafs, H. D.: Sperm output of rabbits at various ejaculation frequencies and their use in the design of experiments. J. Reprod. Fertil. 15: 27–32, 1968.

36. Amann, R. P., Kavanaugh, J. F., Griel, L. C., Jr. and Voglmayr, T. K.: Sperm production of Holstein bulls determined from testicular spermatid reserves, after cannulation of rete testis or vas deferens, and by daily ejaculation. J. Dairy Sci. 57: 93–99, 1974.

37. Katz, D. F. and Overstreet, J. W.: Sperm motility assessment by videomicrography. Fertil. Steril. 35(2): 188–193, 1981.

38. Overstreet, J. W., Katz, D. F., Hanson, F. W. and Fonseca, J. R.: A simple, inexpensive method for objective assessment of human sperm movement characteristics. Fertil. Steril. 31: 162–172, 1979.

39. O'Connor, M. T., Amann, R. P. and Saacke, R. G.: Comparisons of computer evaluations of spermatozoal motility with standard laboratory tests and their use for predicting fertility. J. Anim. Sci.; in press, 1981.

40. Seidel, G. E., Jr. and Foote, R. H.: Variance components of semen crite from bulls ejaculated frequently and their use in experimental design. J. Dairy Sci. 56: 399–405, 1973.

41. MacLeod, J. and Gold, R. Z.: The male factor in fertility and infertility. II. Spermatozoan counts in 1000 men of known fertility and in 1000 cases of infertile marriage. J. Urol. 66: 436–449, 1951.

42. Freund, M.: Standards for the rating of human sperm morphology. Int. J. Fertil. 11: 97–180, 1966.

43. Belsey, M. A., Eliasson, R., Gallegos, A. J., Moghissi, K. S., Paulsen, C. A. and Prasad, M. R. N.: Laboratory Manual for the Examination of Human

Semen and Semen-Cervical Mucus Interaction, Press Concern: Singapore; 43 pp., 1980.

44. Eliasson, R.: Analyses of semen. In: Progress in Infertility, S. J. Behrman and R. W. Kistnes, Eds., Little Brown and Co.: Boston; pp. 691–713, 1975.

45. Hammerstedt, R. H.: Monitoring the metabolic rate of germ cells and sperm. In: Reproductive Processes and Contraception, K. W. McKerns, Ed., Plenum Publishing: New York; pp. 353–391, 1981.

46. Thorell, J. I. and Larson, S. M.: Radioimmunoassay and Related Techniques. C. V. Mosby Co.: St. Louis; 1978.

47. Gomes, W. R.: Chemical agents affecting testicular function and male fertility. In: The Testis, Vol. 3, A. Johnson, W. Gomes, and N. Van Demark, Eds., Academic Press, Inc.: New York; pp. 483–554, 1970.

48. Jouannet, P., Czyglik, F., David, G., Mayaux, M. J., Spira, A., Moscato, M. L. and Schwartz, D.: Study of a group of 484 fertile men. I. Distribution of semen characteristics. Inter. J. Androl. 4: 440–449, 1981.

49. Schwartz, D., Mayaux, M. J., Spira, A., Moscato, M. L., Jouannet, P., Czyglik, F. and David, G.: Study of a group of 484 fertile men. II. Relation between age (20–50) and semen characteristics. Inter. J. Androl. 4: 450–456, 1981.

50. Heuchel, E., Schwartz, D. and Price, W.: Within-subject variability and the importance of abstinence period for sperm count, semen volume, and pre-freeze and post-thaw motility. Andrologia 13: 479–485, 1981.

51. Rogers, B. J., Van Campen, H., Veno, M., Lambert, H., Bronson, R. and Hale, R.: Relationship between morphology and human-sperm fertilizing ability. Fertil. Steril. 32: 664–670, 1979.

52. Snyder, P. J., Lipschultz, L. I. and Greenberg, S. H.: In: The Testis of Normal and Infertile Men, P. Troen and H. Nankin, Eds., Raven Press: New York; 539 pp., 1977.

53. Yanagimachi, R., Lopata, A., Odom, C. B., Bronson, R. A., Malin, C. A. and Nicolson, G. L.: Retention of biologic characteristics of zona pellucida in highly concentrated salt solution—use of salt-stored eggs for assessing the fertilizing-capacity of spermatozoa. Fertil. Steril. 31(5): 562–574, 1979.

54. Yanagimachi, R., Yanagimachi, H. and Rogers, B. J.: Use of zona-free animal ova as a test-system for assessment of fertilizing-capacity of human spermatozoa. Biol. Reprod. 15(4): 471–476, 1976.

55. Blazak, W. F., Overstreet, J. W., Katz, D. F. and Hanson, F. W.: A competitive *in vitro* assay of human sperm fertilizing ability utilizing contrasting fluorescent sperm markers. J. Androl; in press, 1982.

56. Federal Register: Proposed guidelines for mutagenicity risks assessments. RP FRL 1563-2. 45(221): 74984–74988, 1980.

57. Kapp, R. W., Jr.: Detection of aneuploidy in human sperm. Environ. Health Perspect. 31: 27–31, 1979.

58. Beatty, R. A.: F-bodies as Y-chromosome markers in mature sperm heads: a quantitative approach. Cytogenet. Cell. Genet. 18: 33–49, 1977.

59. Roberts, A. M. and Goodall, H.: Y-chromosome visibility in quinacrine-stained human spermatozoa. Nature 262: 493–494, 1976.

60. Kapp, R. W., Jr. and Jacobson, C. B.: Analysis of human spermatozoa for Y chromosomal nondisjunction. Teratogen. Carcinogen. Mutagen. 1: 193–211, 1980.

II. GLOSSARY OF TERMS USED IN MALE REPRODUCTION

androgen—a class of steroid hormones produced in the gonads and adrenal cortex that regulate masculine sexual characteristics; a generic term for agents that encourage the development of or prevent changes in male sex characteristics.

backward motility—the movement of a sperm in a reverse direction (toward the middle piece) rather than a forward direction. Note: backward motility is typically caused by a 180° reflection of the middle piece, which may be a secondary abnormality or may be an artifact induced by temperature shock or osmotic shock.

cellular association or **stage**—one of a series of characteristic cellular groupings of different types of germ cells found in a specific area of a seminiferous tubule. Each association contains several layers of germ cells, each layer representing one cell generation. These groupings are not random. Thus, each association contains specific germ cell types in certain developmental phases. For example, spermatogonia of a specific type are always found with primary spermatocytes of a certain developmental phase and spermatids of a certain developmental phase. One cellular association or stage is found at any moment in a given site within a tubule. Cellular associations are a consequence of the synchronous evolution of the different germ cell generations.

circular motility—a clearly discernible motion at a moderate-to-high velocity, but in circles rather than a more or less linear direction.

cohort of germ cells—all germ cells that are the progency of one A-spermatogonium. Since cytokinesis is incomplete, all germ cells in the cohort remain joined by intercellular bridges and develop synchronously.

cycle of the seminiferous epithelium—the complete series of cellular associations occurring in the seminiferous epithelium (6 stages in man; 14 stages in the rat; and generally classified into 8 stages in the rabbit).

daily spermatozoal output—the total number of sperm ejaculated over an interval of at least 7 days after depletion of epididymal reserves, expressed on a per-day basis. Note: for males ejaculating once every 1 to 3 days, after the reserves of spermatozoa in the cauda epididymidis and ductus deferens have been stabilized, daily spermatozoal output will approach daily spermatozoal production.

daily spermatozoal production—the total number of sperm produced per day by the two testes.

duration of spermatogenesis—the interval between the time a stem spermatogonium becomes committed to produce a cohort of spermatids and the release of the resulting spermatozoa from the germinal epithelium. It is likely that the duration of spermatogenesis requires between 4.3 and 4.7 cycles of the seminiferous epithelium (exact values for most species are unknown). It is difficult to establish the time interval between formation of the stem spermatogonium and formation of preleptotene primary spermatocytes, but this interval may equal the duration of between 1.2 and 1.7 cycles of the seminiferous epithelium in many species. Therefore, the term amputated spermatogenesis is occasionally used to refer to the portion extending from formation of the preleptotene spermatocytes through spermiation; this process typically requires about three cycles of the seminiferous epithelium. The entire duration of spermatogenesis would total 4.2 to 4.7 cycles of the seminiferous epithelium (about 72 days in the human and fewer in most animals).

duration of the cycle of the seminiferous epithelium—the interval required for a cell to pass through one complete series of cellular associations. This duration is constant for a strain or species (12.9 days for Wistar rat, 10.7 days for rabbit, and 16.0 days for human). The cycle length is unaffected by environment, hormonal levels, or cytotoxic damage to the germ cells.

efficiency of spermatozoal production—the number of sperm produced per day per gram of testicular parenchyma.

ejaculate—the total seminal sample obtained during ejaculation.

ejaculation—the expulsion of semen through the urethra.

emission—deposition of sperm and fluids from the caudae epididymidis and ductuli deferentia and fluids from the accessory sex glands into the pelvic urethra.

flagellating spermatozoon—a sperm (not stuck to the glass slide) whose position does not change, although its tail moves back and forth.

follicle-stimulating hormone or **FSH**—a glycoprotein hormone secreted by the anterior pituitary of vertebrates that promotes spermatogenesis and stimulates growth and secretion of the Graafian follicle.

luteinizing hormone or **LH**—glycoprotein hormone secreted by the adenohypophysis of vertebrates that stimulates hormone production by interstitial cells of gonads.

maximum tolerated dose (MTD)—the highest dose that can be given during a chronic study without a possibility of shortening an animal's life other than through its carcinogenicity.

meiosis—two divisions of primary spermatocytes to first form secondary spermatocytes and secondly to form spermatids. Cells are called primary or secondary spermatocytes.

nonmotile spermatozoon—a sperm that does not quiver or move a discernible distance during visual observation.

percentage of motile sperm—the percentage of sperm that are progressively motile, circularly motile, or backward motile; conventionally estimated as a subjective observation of sperm in a diluted sample of semen viewed with a phase-contrast microscope. Note: this percentage can be determined objectively using one of several procedures.

percentage of progressively motile sperm—the percentage of sperm that are progressively motile (excluding circularly motile and backward motile sperm); conventionally estimated as a subjective observation of sperm in a diluted sample of semen viewed with a phase-contrast microscope. Note: this percentage can be determined objectively using one of several procedures.

primary abnormality—an abnormality of sperm morphology originating during spermatogenesis, often associated with the head. Fertilization of an ovum by the spermatozoon characterized by a primary abnormality is unlikely.

progressive motility—a clearly discernible, fairly continuous, forward motion at a moderate-to-high velocity in a reasonably linear path. Progressive motility is greater than 25 μm/sec for human sperm. Note: in nonfrozen semen, many sperm will rotate on their long axis while swimming progressively, although in frozen-thawed semen, progressive motility may not be accompanied by cellular rotation. Also, the composition of the buffer used to dilute a sample of semen can influence whether a motile sperm will rotate or swim without rotation (flat). Rotation about the long axis and the helical beat of the tail often move the head of the sperm in a zig-zag path rather than a true linear path.

quantitative evaluation or **objective evaluation**—an analytical measurement of sperm motility or velocity performed by a nonbiased instrument rather than visually by an individual.

quivering spermatozoon—a sperm that rotates slightly on its long axis or oscillates; characteristic of some sperm recovered from the efferent ducts or rete testis.

secondary abnormality—an abnormality of sperm morphology induced during epididymal transit or ejaculation, usually associated with the tail. When a secondary abnormality is induced in a spermatozoon, its competitive ability to fertilize an ovum is reduced.

semen—a mixture of sperm and fluids from the excurrent ducts and accessory sex glands.

seminal volume—the volume of an ejaculate (expressed in milliliters).

seminiferous epithelium—the normal cellular components within the seminiferous tubule consisting of Sertoli cells and germ cells (spermatogonia, primary spermatocytes, secondary spermatocytes, and spermatids). Sertoli cells are somatic cells that are usually nondividing in adult animals and probably are important for metabolic exchange between the germ cells in the luminal compartment and that, by means of Sertoli-Sertoli junctions, form the blood-testis barrier. They also aid in coordination of spermatogenesis and have an endocrine function.

spermatogenesis—the sum of the transformations that result in formation of spermatozoa from spermatogonia and continued formation of a fairly constant number of uncommitted spermatogonia. The entire spermatogenic process is initiated in early embryonic development and continues after birth and puberty as a consequence of continual renewal of stem cells. At birth two cell types are found within the seminiferous tubule: supporting cells, which give rise to the Sertoli cells of the puberal male, and the gonocytes, which will develop into spermatogonia. The intense proliferation of germ cells and the subsequent release of spermatozoa do not occur randomly. Rather the germinal elements always follow the same pattern of development (unless particular cells and their progeny degenerate) within males of a species.

spermatozoal velocity—the velocity with which a progressively motile or circularly motile sperm moves. Spermatozoal velocity is conventionally expressed on a subjective scale from 0 (low velocity) to 4 (maximum velocity) but should be expressed as μm/sec on the basis of quantitative measurements.

spermiation—release of spermatozoa from the germinal epithelium into the lumen of the seminiferous tubule. Prior to release the germ cells are called spermatids, and after spermiation they are called spermatozoa.

spermiogenesis—the differentiation of spermatids from spherical cells with considerable cytoplasm to characteristically shaped cells with a highly condensed nucleus and scant cytoplasm but with a flagellum. Cells are called spermatids. Based on changes in the spermatid acrosome, spermiogenesis can be considered as a continuum consisting of four phases: Golgi, cap, acrosome, and maturation. In addition to acrosomal evolution, condensation of the nuclear material and formation of the flagellum occur.

subjective evaluation—a visual estimate subject to observer bias and error.

testosterone—a biologically potent androgenic steroid that may be released from the gonads and adrenal glands.

total spermatozoa per ejaculate—the total number of spermatozoa in an ejaculate (determined as the product of seminal volume times spermatozoal concentration and expressed as 10^6). Note: the total number of sperm per ejaculate, not spermatozoal concentration, provides the best information on the number of spermatozoa produced by the testes, since spermatozoal concentration is influenced by the relative contributions of the accessory sex glands diluting the bolus(es) of sperm transported during emission from the ductus deferens and cauda epididymidis.

twitching spermatozoon—a sperm that occasionally or continuously moves a short distance with a violent motion and then comes to rest, at least momentarily, before an additional twitch or jump. The twitch or jump need not be in a forward direction.

CHAPTER 4

CURRENT STATUS OF, AND CONSIDERATIONS FOR, ESTIMATION OF RISK TO THE HUMAN CONCEPTUS FROM ENVIRONMENTAL CHEMICALS

Definition and Scope

Teratology is the study of the causes, mechanisms, and sequelae of perturbed developmental events in species of animals that undergo ontogenesis. This report is restricted to a consideration of factors influencing the current status of risk assessment of teratologic effects of environmental agents. It is considered a preliminary document touching upon the major considerations basic to quantitative estimation of risk to development of the conceptus following exposure of pregnant animals to environmental agents. This document provides no definitive means for assessing risks to the human conceptus, since no documented or validated system for such assessment has yet been established. Basic to risk estimation is hazard assessment, which requires quantification and validation of reliable end point assays. This document briefly discusses the factors and scientific considerations upon which degrees of confidence applicable to contemporary studies of teratology are to be based. Some additional considerations in evaluating experimental data (e.g., acute versus chronic exposures) have not been covered explicitly, but references are provided to aid the reader in gathering further information.

Impact of Developmental Abnormalities on Humans

Approximately 50% of human conceptuses fail to reach term, and perhaps as many as half of those lost are structurally abnormal (1). Approximately 3% of newborn children are found to have one or more significant congenital malformations at birth, and by the end of the first postnatal year, approximately 3% more (2, 3) are found to have developmental malformations. An additional group, whose size is difficult to estimate, has functional abnormalities of the nervous, respiratory, gastrointestinal, immunologic, and other systems. Some unknown proportion of these abnormalities may be due to environmental insult during prenatal life.

Causes of Congenital Malformations

Relatively little is known about the specific causes of most human congenital defects. It is estimated that 10 to 15% of all human congenital malformations are due to environmental agents and another 10 to 15% to hereditary factors (i.e., gene mutations and chromosomal aberrations). The remainder are considered to result from unknown causes and from complex interactions between multifactorially determined hereditary susceptibilities and microenvironmental factors precipitating abnormal developmental sequences within the conceptus and its associated membranes. To date, only a relatively small number of specific environmental agents and factors have been identified as causing human malformations (4).

From the above, it is concluded that although regulatory controls on man-made environmental agents may reduce the incidence of developmental abnormalities, they will not totally prevent them. It must be recognized that indications from animal experiments of adverse effects of environmental agents on development may not always be corroborated by observations of perturbed development in human populations. Nevertheless, and in full recognition of these qualifiers, standard animal testing is presently considered the best available method for predicting risk of congenital malformation in human beings prior to human exposure. Information derived from such testing can be used to detect and to estimate the magnitude of hazard posed by specific substances to human prenatal development and can serve as a basis for estimation of risk.

Qualitative Evaluation of Risk Potential

Interspecies comparisons

Inherent interspecies differences complicate extrapolation of animal test results to direct determination of human risk. Because a species identical to the human in all relevant characteristics does not exist, interspecies differences between human beings and the test species must be considered when data are being evaluated. Interpretation of these inherent interspecies differences is complicated by species differences in metabolism and pharmacokinetics of the test agent and in developmental and other attributes characteristic of the species. Very little is currently understood about the extent and nature of the interplay among these many factors as they may affect the production of a teratogenic event.

Since human beings are manifestly heterogeneous, there is little doubt that human populations will contain broad degrees of susceptibility and resistance to the possible adverse prenatal effects of environmental agents. Because this heterogeneity is largely determined by genetic variability, it has been reasoned that stocks of animals bred at random are the most appropriate models for testing teratogenicity. However, in order to estimate the degrees of susceptibility that may exist within human populations, both the average response of the test group and the extent of responses within it must be considered. This goal can be achieved to some extent by using several stocks of animals. To make such a procedure even more sensitive and useful, several inbred strains may also be tested, since this procedure increases the likelihood that a range of sensitivities will be uncovered (5). For instance, genetically controlled variations in embryonic face formation account partly for the sensitivity of certain mouse strains to spontaneous (6) and teratogen-induced (7, 8) cleft lip and isolated cleft palate.

Dosing and mode of administration

The test agent should be administered over a range of doses, including a level sufficient to produce signs of maternal toxicity in the particular species used. If a teratogenic response is observed, a dose-response relationship should be determined for the agent and that specific teratogenic effect. In using test animals, the selection of dosing intervals must take into account the varying degrees of sensitivity during organogenesis in that species, the possibility of enzyme induction or other modifying processes that could result from repeated administration of the test material, and the practical aspects of administration that would make the dosing comparable to that which would likely occur with human exposure.

The route of administration of the test agent may significantly affect the outcome of an experiment. In general, the route of exposure for test animals should mimic that of human exposure where possible, although valuable data may be obtained from other routes of exposure as well. Differences in the response of a species to the route, dose, and vehicle used for exposure to the test agent may result in significant variations in blood and tissue levels of the agent in the maternal and embryo-fetal units (9). These factors may or may not be of direct significance to teratogenesis, but they must be recognized as being potentially significant.

Placental transfer

The anatomy and physiology of the placentas of experimental animals and man present a diverse spectrum of maternal-fetal connections (10). The chorioallantoic placenta of the human is approximated by that in some non-human primates, whereas the common experimental animals have, in addition, a yolk sac placenta, which also structurally and functionally joins embryo and mother. The extent to which the yolk sac may supplement or complement transfer of a previously untested chemical via the chorioallantoic circulation is largely unpredictable. In most of these species (e.g., rat, mouse, rabbit, guinea pig), the yolk sac placenta may play a major role in maternal-fetal exchange of substances during early organogenesis. The chorioallantoic placenta, which is readily available for convenient study at term, is in most cases a totally different structure from that effecting transfer during the critical stages of development; therefore great care must be exercised to avoid unwarranted extrapolation from studies of term chorioallantoic placenta to presumptions for the function of the two placental structures present earlier in gestation. Lipid solubility, ionic charge, molecular size, and specific structural configuration all appear to contribute to the transfer of chemicals between mother and fetus. Little or no relationship may exist between the embryonic and fetal concentration of agents and their possible teratologic effect, since potent teratogens do not always accumulate in the fetus at concentrations greater than those of agents with low teratogenic potential. Consequently, increased concentration ratios between the conceptus and mother do not necessarily allow predictability of teratogenic or other embryotoxic potential (11, 12). Little is currently known about the sites of action of teratogenic agents; therefore, any component of the entire maternal-placental–embryo-fetal unit and all combinations of such should be considered as possible site(s) for teratogenic action, until the mechanism of teratogenic action for given agents is better understood.

Pharmacokinetics and metabolism

Other variables that may affect the teratogenicity of an agent in various species include pharmacokinetics and metabolism and those exogenous factors that may affect these parameters. Some of the factors to be considered when evaluating data from these experiments are seasonal and circadian effects on development and metabolism (13, 14); interaction of pharmacokinetic and placental hemodynamics (15); possible sites of action of agents; sites of maternal, placental, and embryonic-fetal metabolism; and deposition and/or depression or induction of metabolic enzymes (9, 16). Certain agents may stimulate or inhibit enzyme systems, such as liver microsomal enzyme systems. In some cases single doses at critical periods in gestation induce a greater teratologic response than divided doses on several consecutive days (17–21). Maternal and embryo-fetal nutritional and endocrine states in various species may interact with and/or alter metabolism and pharmacokinetics (22), as may species-specific effects resulting from repeated administration of the test agent, saturation of metabolizing enzymes, and inhibition or induction of biotransforming enzymes (16, 23).

Basic to interpreting data from studies of teratology is documentation of a dose-response relationship and determination of a treatment level below which adverse effects are not evident in the data available (no-observed-effect level or NOEL). Threshold levels may be encountered, and dose levels can exist below which development of the conceptus suffers no observable deleterious effect at term. The no-observed-effect level does not guarantee absolute safety, because uncertainty may result from biological and/or statistical variation. Failure to detect a deleterious effect on the end point examined could indicate the absence of a deleterious effect, but absence of observed effect also could occur if the magnitude of an effect were below the limit of statistical detection ability.

Mechanisms of action

The mechanisms underlying abnormal embryonic development are not well understood. It has proven difficult to determine whether an observed incidence of abnormal development is the result of an agent or one of its products acting directly on the conceptus or its placenta, or if it is achieved indirectly through an initial effect on the mother. Therefore, the primary site of action by an agent capable of disrupting development may or may not be the specific malformed organ and may not even be within the conceptus. Whether or not a chemical is evenly distributed within the mother and conceptus, its action on a particular tissue or organ may be dependent on cellular interactions and the particular developmental events characteristic of specific ontogenetic stages. Increased knowledge of these ontogenetic events and the interaction of toxic agents or their products with them is needed both to understand the resulting defects and to enable better extrapolation of effects seen in one species to predictions of potential effects in another.

Animal Studies

Standard teratogenicity testing

Schardein (24) has discussed in some detail the current methodology and testing approach initially outlined in the 1966 Food and Drug Administration's (FDA's) guidelines for reproductive studies and in the 1967 and 1978 World Health Organization's recommendations, which are further specified in the U.S. Environmental Protection Agency's proposed guidelines. Numerous countries have required studies that are generally similar but that vary in particulars. The object of the standard protocols is to expose animals to test materials before breeding of the parental generation, during *in utero* development and lactation, and in some instances into adult life of the offspring. To achieve their goals, the experiments are designed in three phases or segments, with a multigeneration test for reproductive effects required in some instances.

The first phase, or Segment-I protocol, calls for dosing of both male and female animals to begin some calculated time prior to breeding. Treatment of the young males begins 60 days prior to breeding, and exposure of the females to the test substance begins two weeks prior to breeding. Dosing continues for both sexes during the breeding interval and for the impregnated females throughout pregnancy and lactation. Other major details of the Segment-I protocol could be described here, but these will be slighted t emphasize the basics.

The Segment-I protocol is supposed to examine for possible adverse effects on estrus; sexual performance; formation of the gametes; their release from the gonad, transport, and interaction to form a zygote; and zygote passage to and implantation into the decidua. Because dosing of the dam continues after mating, the protocol could reveal effects on placental formation and its function; and because dosing continues throughout gestation and lactation, the protocol could reveal adverse effects on embryonic or fetal development and on delivery, nurture, and postnatal development of the pups. Adverse effects of the test material on the supportive functional parameters essential for normal occurrence of each of the above could also become evident. These effects could be as diverse as effects on food intake or altered endocrine status. This study is usually made in rats, and by indicating problem areas, it can serve as a preliminary to later studies.

The second protocol is oriented more specifically to detect effects on embryonic development. The study is usually made in both rats and rabbits. The Segment-II study requires that the males not be treated with the test compound and that treatment of the pregnant females not begin until after decidual implantation of the blastocysts has occurred. Treatment ceases at the end of major organogenesis, usually considered as the time of closure of the secondary palate in the species. Autopsy is performed the day before expected delivery, when the term fetuses are collected for gross external examination, after which they are examined for skeletal development and internal soft-tissue morphology.

The goal of this experiment is to detect adverse effects of a test material on the developmental events characteristic of major organogenesis in the embryo.

A Segment-III evaluation is a perinatal and postnatal study requiring treatment of the dams only. The test agent is administered during the last third of pregnancy and throughout lactation. Treatment is not scheduled to begin until after the period of major embryonic development is completed.

The Segment-III safety evaluation was designed to detect adverse effects of substances on fetal development as well as those developmental processes that continue into infancy and adolescence. It is in this study that potential effects on postnatal behavior of the young are usually evaluated.

A rather elaborate multigeneration protocol is employed for evaluating selected substances for effects on reproduction over three enerations. The goal of the multigeneration protocol is to reveal effects caused by accumulated toxicity or by agents effective at low concentration. These protocols for safety evaluation have had detailed discussion (14, 25), and for each a data base of considerable size has accumulated. They are not considered as final, however, because there is a need for flexibility and exercise of scientific judgment (26), which could improve their detecting ability in some instances. There is also some justification for their revision in light of research findings in related fields since their inception.

There are four types of developmental defects: gross anatomical, death in utero, growth retardation, and functional deficit (4). Currently, the first three of these end points are the only ones that have a data base sufficient to ensure confidence in their applicability for use in regulatory decisions. Functional status has been studied broadly only in recent years and soon may develop end point assays with specific applicability.

Examination of fetuses to identify gross anatomical defects often entails judgmental and subjective appraisals based on criteria or standards established by individual laboratories. The routine test as performed by many laboratories applying FDA's Good Laboratory Practices requires highly trained technical and professional personnel. Even though general standards for defining the limits of normality and associated terminology have not been established, in general when selected compounds have been evaluated by various laboratories, similar findings have been demonstrated by those using the routine teratology test and its methods for examining the young (27, 28).

The overwhelming majority of chemicals known to be teratogenic in human beings have been demonstrated to be teratogenic in one or more common laboratory species. Many other agents shown to be teratogenic in laboratory animals have not yet been documented as teratogens in humans. The difference may be due to insufficient epidemiologic data, dissimilarities of exposure levels, or differences in end points analyzed. A teratogenic response in one species or strain should be considered indicative of a potential teratogenic hazard for human beings. However, negative responses in a few species of experimental animals do not necessarily guarantee absence of adverse effects in human conceptuses. In the routine teratology test, no one species has been consistently more predictive of human teratogenicity than another species.

Functional teratogenicity testing

Functional alterations may prove to be sensitive indicators of teratogenic potential. Among those that have been studied following prenatal exposure, a broad and complex range of behavioral effects has been described. There is concern that these effects may occur at doses below those producing gross structural defects or prenatal death (29). Current literature is based largely on studies of rodents, particularly rats, in which it has been demonstrated that exposure to chemicals during periods from early organogenesis through pubescence can result in behavioral impairments. To detect such effects more efficiently, reliable and sensitive test procedures are being developed in several laboratories (30, 31). Although there are no agreed-upon testing methods, current studies routinely include the end points in the following areas: (a) reflex ontogeny, (b) habitation and reactivity, (c) learning and problem solving, (d) activity level, (e) motor skills, and (f) sensory processes.

Other functional parameters demonstrated to be affected by prenatal exposure to chemicals include fertility; reproduction; the endocrine system; immune competence; xenobiotic metabolism; and various physiologic parameters, including cardiovascular, renal, gastrointestinal, respiratory, and hepatic functions (32). Finally, late sequelae of prenatal exposure to chemicals may be manifested postnatally as cancers or shortened life span (33, 34).

Gross structural defects or significant growth retardation may complicate analysis of data from tests of function, and alertness to potential confounding factors is essential. Permanent changes in functional systems should be viewed as indicating the potential for an adverse effect in human beings. Transient changes or delays in functional ontogeny are still not understood, and their significance must be further evaluated.

Short-Term Testing Procedures

Prioritizing of chemicals for in-depth study

As a prelude to estimation of potential risks, a series of biological and informational factors may be applied to a new substance to possibly trigger further testing to some level in a tier system of evaluations for teratologic effects. It is considered highly desirable that substances be prioritized for testing to focus research attention more readily on substances injuring conceptuses at doses significantly below those toxic to adults. In attempting to list the factors to be taken into consideration, the need for short-term systems became evident because of the large number of evaluations needed and the fact that many of the available data would be in category 2 below. Listed below are the factors that, when applicable, would make a substance a high-priority candidate for further testing. Within each of the two categories, the factors are listed in decreasing order of importance.

1. Biological effects data possibly available regarding a substance: Suspected human teratogenicity; Teratogenicity in domestic

animals or wildlife established; Short-term teratology test indicating a significant developmental hazard potential; Adult toxic dose/developmental toxic dose ratio large; Toxicity documented in the adult at low dosage;

2. Additional information available regarding a substance: Large numbers of women exposed; Bioaccumulation evident; Persistence of substance in environment; New substance; Involuntary exposure.

The number of agents in use and potentially impinging on human development is already vast and is increasing rapidly. Some unknown small fraction of these may be potentially harmful to human conceptuses at doses below those obviously deleterious to adults. Short-duration and low-cost methods for detecting and prioritizing those substances posing the greatest potential hazard to the conceptus are needed. Because standard tests in animals are quite costly, only a rather small number of substances of potential teratogenic risk can be evaluated each year. This situation requires development and validation of short-term methods that will permit rapid and meaningful testing of these chemicals. It is necessary to develop, validate, and use assays that will permit more economical testing of a larger number of agents than could be tested rapidly and conveniently by standard teratogenicity evaluations. Validation should consist of various forms of positive correlation between the results of such tests and those found in conventional *in vivo* test procedures. Particular attention should be directed to correlations with known human teratogenic responses whenever reliable data are available. Included should be chemicals already known to have significant hazard potential for the conceptus, as well as chemicals considered as lacking such potential. If properly validated short-term tests were to indicate either potential hazard or safety, such determinations would be helpful for establishing a priority system for further tests aimed toward quantitative risk assessment.

Characteristics of short-term assays

Short-term tests might be considered as either preliminaries to more detailed evaluations, or they might serve as efficient means to detect those substances capable of posing the greatest hazard to the conceptus. Whether they serve either or both of these slightly different goals, the tests should possess certain attributes: they should be rapid, economical, and reproducible from one laboratory to another; should have easily identifiable end points; and ideally should prioritize substances according to their potential for posing hazards to the conceptus. Confidence in their applicability would be increased by demonstration of dose-response relationships. They should give minimal false negatives; it is understood that false positives can be explored further in more elaborate animal testing. Ideally, the system should encompass as many as possible of the developmental events known to occur in the conceptus.

Short-term tests may serve as preliminary screens to aid in the detection of possibly teratogenic hazards. To accomplish this, several such tests would probably have to be performed concurrently or sequentially. It must be remembered that such indications of teratogenic hazard potential must be used prudently for estimating risk to human beings. Risk estimation can only be achieved by the use of systems that have been extensively validated, and to date, only the more routine standard testing methods are considered applicable to this use.

Potential short-term systems

In vivo. Two *in vivo* systems have been advanced as possible short-term systems. One is an abbreviated version of a standard teratology test protocol using the maternal maximum tolerated dose and neonatal evaluation shortly after birth (35). The second is evaluation of homeotic shifts that may prove effective for detecting minimal expression of teratogenic hazard potential (36). Each system has potential merit, but as in the *in vitro* systems, needs careful peer review or detailed validation.

In vitro. Artificial invertebrate "embryos," embryonic insect cells, amphibian and fish embryos, or organs of avian and mammalian embryos (palatal shelves, tooth bud, kidney mesenchyme, pancreas, bone primordia, lens, sex organs, etc.) have the potential to serve as the basis for *in vitro* systems. Table 12 lists a number of potential *in vitro* systems. In these procedures, cells, organs, or whole embryos have been exposed to various chemicals and their effects measured with the end point permitted by each system. When attempted in a few instances, adequate dose-response relationships were obtained by some systems. In their present state, the systems listed in Table 12 have been only partially validated (36–50), and their closer examination is necessary.

TABLE 12 Some *In Vitro* Short-Term Systems Currently in Various Stages of Development

System		Developmental parameters monitored	References
Invertebrates	Hydra	Various	36
	Planaria	Regeneration, dose-response relationship of developmental toxicity	37, 38
	Drosophila cells	Differentiation	39
Fish	Zebra fish	Dose-response relationship of developmental toxicity	40
Vertebrate cell culture	Chick embryo neural crest	Morphology, differentiation	41
	Chick embryo limb bud mesenchyme	Differentiation	41
	Mouse tumor cells	Cell attachment	42
	Teratocarcinoma stem cells	Differentiation (*in vivo* or *in vitro*)	43
Organ culture	Mouse embryo limb bud	Growth, dysmorphogenesis, differentiation	44, 45 46, 47
Whole embryo culture	Rat, mouse, chick	Growth, dysmorphogenesis, histogenesis	16, 48 49, 50

Quantitative Risk Assessment

Quantitative risk assessment is based on the relationship between laboratory findings and expected human response. If an agent demonstrates teratogenicity in any mammalian species, some concern about prenatal human exposure to the agent is justified. The level of concern is to be tempered by numerous considerations, not the least of which is the extent of maternal toxicity evident at the dose level needed to elicit a toxic response in the conceptus (51). It is assumed that margins of safety applied to the experimental data in test species can be used to estimate an allowable exposure in pregnant women. It is considered that no-observable-effect and/or threshold levels of exposure do exist (52) for at least some teratogens. The determination of human risk requires the definition of moderating (or modulating) conditions such as the distribution of the compound within the environment; its pattern of use and exposure (whether intermittent or chronic); and the identification of those subpopulations that may be at high risk as a result of factors such as life style, age, occupation, etc.

The use of animal test systems under highly controlled experimental conditions has conditional validity for defining human risk. Although only warning systems at best, laboratory experiments can provide, in addition to the factors already mentioned, two types of information that may be useful for estimation of the potential human risk. These are (a) the ratio of the adult and developmental toxic doses and (b) the shape of the curve of the teratogenic dose response. Although not markedly informative to date, more detailed delineation of projected effects may be obtained through use of pharmacokinetic information, focusing on access of the agent to relevant site(s) of teratogenic action (53). However, knowledge of teratogenic mechanisms and identification of the sites actually relevant must be obtained before these considerations can achieve their full potential utility.

It is often necessary to conduct animal experiments at dosage levels exceeding estimated levels of human exposure to increase the likelihood that a weak teratogen will produce an apparent effect and to compensate for the relatively small numbers of animals used in the test. This requires extrapolation of results from experimental dosage levels to lower levels of human exposure. There is no uniform basis for selecting the appropriate mathematical model for such extrapolation.

Safety factors may be applied to establish acceptable dosage levels that are expected to yield acceptable levels of risk. The size of the safety factor depends upon the quality and quantity of the biological effects data available.

For many biological systems, the dose-response curve tends to flatten at low doses, and for some teratogens this is an important consideration (54). Hence, decreasing dosage by a safety factor of F will generally decrease risk by more than a factor of F. That is, if the upper confidence limit on the risk is U at an experimental dosage of d, the potential risk at a lower dosage of d/F is predicted to be less than U/F for the test animal. The uniformity with which this would apply to potential teratogenic hazards is undetermined as is the degree of interspecies uniformity for the difference between the adult (A) toxic and developmental (D) toxic doses. Presence or absence of uniformity in the A/D ratio or slope of the dose-response between studies in different species would also influence the magnitude of safety factors.

Priorities for Future Research in Teratology

The areas of research in teratology recommended below focus on two broad objectives: (a) development of practical and informative testing systems with which to evaluate both the plethora of chemicals now in existence and those yet to be developed and (b) scientific advancement in teratology so that the currently employed and largely standard test systems can become more useful and reliable for human risk estimation. The second objective does not imply that currently available methods cannot be used for human risk estimation. An opposite view is held, and within limits, such estimations are possible at the present time on the basis of data from current state-of-the-art studies. It is felt, however, that methods are needed to identify more rapidly those chemicals potentially the most hazardous and to expand the understanding and applicability of all test methods.

1. The degree to which the end point determinations of adverse effects on development encountered in the standard protocols predict adverse effects in other species (especially humans) has not been reported in detail sufficient for precise quantification of human risk. Such studies are encouraged as are those that may indicate how the predictive ability of tests in animals can more precisely herald human responses.
2. Validated methods are needed for rapid and inexpensive detection of substances uniquely toxic to conceptuses (i.e., substances that are embryotoxic at doses below those producing adult toxicity).
3. A better understanding of mechanisms of teratogenic action or elucidation of the steps in the pathway between exposure and effect might significantly improve and refine end point assays. Similar studies of pathogenesis are needed for effects on biochemical and physiological systems in the dam, uterus, and placenta.
4. Development of a broader data base on comparative metabolism and pharmacokinetics correlated with teratologic end points may eventually enhance ability to make interspecies extrapolations.
5. There is a need to develop and validate methods to detect and quantify possible functional impairments.
6. Methods are needed to detect and predict additive or synergistic effects more effectively.

REFERENCES

1. Hertig, A. T.: The overall problem in man. In: Comparative Aspects of Reproductive Failure, K. Benirschke, Ed., Springer-Verlag: New York; pp. 11–41, 1967.
2. McKeown, T. and Record, R. C.: Malformations in a population observed for five years after birth. In: Ciba Foundation Symposium on Congenital Malformations, G. E. W. Welstenholme and C. M. O'Conner, Eds., Little Brown: Boston; pp. 2–16, 1963.
3. Mellon, G. W. and Kalzenstein, M.: Increased incidence of malformations – chance or change? J. Am. Med. Assoc. 187: 570–573, 1964.
4. Wilson, J. G.: Environment and Birth Defects. Academic Press: New York; 305 pp., 1973.
5. Kalter, H.: Interplay of intrinsic and extrinsic factors. In: Teratology: Principles and Procedures, J. G. Wilson and J. Warkany, Eds., University of Chicago Press: Chicago; pp. 57–80, 1965.
6. Trasler, D. G.: Pathogenesis of cleft lip and its relation to embryonic face shape in A/J and C57BL mice. Teratology 1: 33–49, 1968.
7. Trasler, D. G.: Aspirin-induced cleft lip and other malformations in mice. Lancet 1: 606–607, 1965.
8. Trasler, D. G. and Fraser, F. C.: Time-position relationships with particular reference to cleft lip and cleft palate. In: Handbook of Teratology, Vol. 2, J. G. Wilson and F. C. Fraser, Eds., Plenum Press: New York; pp. 271–292, 1977.
9. Larsson, K. S.: Contributions of teratology to fetal pharmacology. In: Fetal Pharmacology, L. O. Boreus, Ed., Raven Press: New York; pp. 401–418, 1973.
10. Beck, F.: Comparative placental morphology and function. Environ. Health Perspect. 18: 5–12, 1976.
11. Waddell, W. J. and Marlowe, G. C.: Disposition of drugs in the fetus. In: Perinatal Pharmacology and Therapeutics, B. L. Mirkin, Ed., Academic Press: New York; pp. 119–268, 1976.
12. Wilson, J. G., Scott, W. J., Ritter, E. J. and Fradkin, R.: Comparative distribution and embryotoxicity of methotrexate in pregnant rats and rhesus monkeys. Teratology 19: 71–80, 1979.
13. Barr, M., Jr.: Prenatal growth of Wistar rats: circadian periodicity of fetal growth late in gestation. Teratology 7: 283–288, 1973.
14. Layton, W. M.: An analysis of teratogenic testing procedures. In: Congenital Defects, New Directions in Research, D. T. Janerich, R. G. Skalko and I. H. Porter, Eds., Academic Press: New York; pp. 205–217, 1974.
15. Wolkowski-Tyl, R. M.: Strain and tissue differences in cadmium-binding protein in cadmium-treated mice. In: Developmental Toxicology of Energy-related Pollutants, D. D. Mahlum, M. R. Sikov, P. L. Hackett and F. D. Andrew, Eds., Technical Information Center: Oak Ridge, Tennessee; pp. 568–585, 1978.

16. Fantel, A. G., Greenaway, J. C., Juchau, M. R. and Shepard, T. H.: Teratogenic bioactivation of cyclophosphamide *in vitro*. Life Sci. 25: 67–72, 1979.

17. Russell, L. B., Badgett, S. K. and Saylors, C. L.: Comparison of the effects of acute, continuous, and fractionated irradiation during embryonic development. Int. J. Radia. Biol., Suppl: 343–359, 1960.

18. Wilson, J. G.: Effects of acute and chronic treatment with Actinomycin D on pregnancy and the fetus in the rat. Harper Hosp. Bull. 24: 109–118, 1966.

19. King, C. T. G., Horigan, E. and Wilk, A. K.: Fetal outcome from prolonged versus acute drug administration in the pregnant rat. In: Drugs and Fetal Development, M. A. Klingberg, A. A. Abramovici and J. Chemke, Eds., Plenum Press: New York; pp. 61–75, 1972.

20. Tuchmann-Duplessis, H.: Teratogenic Action of Drugs. Pergammon Press: New York; 1965.

21. Belisle, R. J. and Long, S. Y.: Tolbutamide treatment of pregnant mice: repeated administration reduces fetal lethality. Teratology 13: 65–70, 1976.

22. Neubert, D., Merker, H.-J., Kohler, E., Krowke, R. and Barrach, H. J.: Biochemical aspects of teratology. In: Advances in the Biosciences, G. Raspe, Ed., Pergammon Press: Oxford; pp. 575–622, 1971.

23. Bakay, B. and Nyhan, W. L.: Effects of Thalidomide and Chlorcyclizine on the biosynthesis of nucleic acids and proteins in fetal and maternal tissue of the rat. J. Pharmacol. Exp. Ther. 171(1): 109–117, 1970.

24. Schardein, J. L.: Drugs as Teratogens. CRC Press: Cleveland; pp. 9–12, 1976.

25. Kelsey, F. O.: Present guidelines for teratogenicity studies in experimental animals. In: Congenital Defects, New Directions in Research, D. T. Janerich, R. G. Skalko and I. H. Porter, Eds., Academic Press: New York; pp. 195–204, 1974.

26. Golberg, L. M. B.: Discussion pp. 53–55. In: Methods for Detection of Environmental Agents that Produce Congenital Defects, T. H. Shepard, J. R. Miller, and M. Marois, Eds., North-Holland Publishing Co.: Amsterdam; 1975.

27. Wilson, J. G.: Methods for administering agents and detecting malformations in experimental animals. In: Teratology: Principles and Techniques, J. G. Wilson and J. Warkany, Eds., University of Chicago Press: Chicago; pp. 262–277, 1965.

28. Staples, R. E. and Schnell, V. L.: Refinement in rapid clearing technique in the KOH-alizarin-red-S method for fetal bone. Stain Technol. 39: 61–63, 1963.

29. Hutchings, D. E.: Behavioral teratology: embryopathic and behavioral effects of drugs during pregnancy. In: Studies on the Development of Behavior and the Nervous System, Vol. 4, Early Influences, G. Gottlieb, Ed., Academic Press: New York; pp. 7–34, 1978.

30. Vorhees, C. V., Brunner, R. L. and Butcher, R. E.: Psychotropic drugs as behavioral teratogens. Science 205: 1220–1225, 1979.

31. Buelke-Sam, J. and Kimmel, C. A.: Development and standardization of screening methods for behavioral teratology. Teratology 20: 17–29, 1979.

32. Kimmel, C. A.: A profile of developmental toxicity. In: Developmental Toxicology, C. A. Kimmel and J. Buelke-Sam, Eds., Raven Press; in press, 1981.

33. Rice, J. M.: Perinatal period and pregnancy: intervals of high risk for chemical carcinogens. Environ. Health Perspect. 29: 23–27, 1979.

34. Spyker, J. M.: Assessing the impact of low-level chemicals on development: behavioral and latent effects. Fed. Proc. Fed. Am. Soc. Exp. Biol. 34: 1835–1844, 1975.

35. Chernoff, N. and Kavlock, R. J.: A potential *in vivo* screen for the determination of teratogenic effects in mammals. Teratology 21: 33A–34A, 1980.

36. Johnson, E. M.: A subvertebrate system for rapid determination of potential teratogenic hazards. J. Environ. Pathol. Toxicol. 4: 153–156, 1980.

37. Best, J. B., Morita, M., Ragin, J. and Best, J., Jr.: Acute toxic responses of the freshwater planarian, ***Dugesia dorotocephala***, to methyl-mercury. Bull. Environ. Contam. Toxicol.; in press, 1981.

38. Best, J. B., Morita, M. and Abbotts, B.: Acute toxic responses of the freshwater planarian, ***Dugesia dorotocephala***, to chlordane. Bull. Environ. Contam. Toxicol.; in press, 1981.

39. Bournias-Vardiabasis, N., Terplitz, R. L. and Seecof, R. L.: An *in vitro* assay for teratogenesis. Teratology 21: 29A, 1980.

40. Streisinger, G.: Invited discussion on the possible use of zebra fish for the screening of teratogens. In: Methods for Detection of Environmental Agents that Produce Congenital Defects, T. H. Shepard, J. R. Miller and M. Marois, Eds., North-Holland Publishing Co.: Amsterdam; pp. 59–61, 1975.

41. Wilk, A. L., Greenberg, J. H., Horigan, E. A., Pratt, R. M. and Martin, G. R.: Detection of teratogenic compounds using differentiating embryonic cells in culture. *In Vitro* 16: 269–276, 1980.

42. Braun, A. G., Emerson, D. J. and Nichinson, B. B.: Teratogenic drugs inhibit tumor cell attachment to lectin-coated surfaces. Nature 282: 507–509, 1979.

43. Filler, R.: An *in vitro/in vivo* coupled prescreen to identify teratogens requiring metabolic activation. Teratology 21: 37A, 1980.

44. Kochhar, D. M.: The use of *in vitro* procedures in teratology. Teratology 11: 273–288, 1975.

45. Neubert, D. and Barrach, H. J.: Significance of *in vitro* techniques for the evaluation of embryotoxic effects. In: Methods in Prenatal Toxicology: Evaluation of Embryotoxic Effects in Experimental Animals, D. Neubert, H. J. Merker and T. E. Kwasigroch, Eds., Georg Thieme: Stuttgart; pp. 202–209, 1977.

46. Manson, J. M. and Simons, C. R.: *In vitro* metabolism of cyclophosphamide in limb bud culture. Teratology 19: 149–158, 1977.

47. Kochhar, D. M. and Agnish, N. D.: Teratogenic testing *in vitro*. In: Toxicity Testing *In Vitro*, R. M. Nardone, Ed., Academic Press: New York; in press, 1981.

48. New, D. A. T.: Techniques for assessment of teratologic effects: embryo culture. Environ. Health Perspect. 18: 105–110, 1976.

49. Brown, N. A., Goulding, E. H. and Fabro, S.: Ethanol embryotoxicity: direct effects on mammalian embryos *in vitro*. Science 206: 573–575, 1979.

50. Klein, N. W., Vogler, M. A., Chatot, C. L. and Pierro, L. J.: The use of cultured rat embryos to evaluate the teratogenic activity of serum: cadmium and cyclophosphamide. Teratology 21: 199–208, 1980.

51. Johnson, E. M.: Screening for teratogenic potential: are we asking the proper question? Teratology 21: 259, 1980.

52. Staples, R. L.: Teratogens and the Delaney Clause. Science 185: 813, 1974.

53. Young, J. F. and Holson, J. F.: Utility of pharmacokinetics in designing toxicological protocols and improving interspecies extrapolation. J. Environ. Pathol. Toxicol. 2: 169–186, 1978.

54. Jusko, W. J.: Pharmacodynamic principles in chemical teratology: dose-effect relationship. J. Pharmacol. Exp. Ther. 183: 469–480, 1972.

CHAPTER 5

OTHER CONSIDERATIONS: EPIDEMIOLOGY, PHARMACOKINETICS, AND SEXUAL BEHAVIOR

Epidemiology: Methods and Limitations

Epidemiology has been defined as the study of the distribution and determinants of disease and injury in humans. It focuses on the occurrence of disease in groups of individuals or populations rather than in any single individual (1). Ideally, reproductive and teratologic effects of environmental agents would be assessed in epidemiologic studies of human populations because of the difficulties inherent in extrapolating from other species. However, ethical considerations render randomized controlled trials generally unfeasible. If one cannot experiment, then one can only observe, but in some circumstances even observation is not possible (e.g., risk assessment of new chemicals before they are introduced into the human environment).

Observational epidemiologic studies can be classified into those that generate hypotheses and those that formally test hypotheses and quantify risks. The following list is not an exhaustive delineation of all possible epidemiologic approaches in these categories, but it includes those which may be most useful for environmental risk assessment.

Hypothesis–generating studies

Case reports are a source for raising suspicions about substances that might be teratogens or reproductive hazards (e.g., an astute clinician's association of thalidomide with phocomelia). However, only very striking or very rare outcomes can be detected in this manner and often after a considerable length of time. For the vast majority of pregnancy outcomes, formal epidemiologic studies involving appropriate comparison groups are required.

Correlational studies evaluate the patterns of morbidity or mortality in populations where classification is made on the basis of aggregates of individuals as distinct from single individuals (e.g., geographic-specific spontaneous abortion rates correlated with area-specific air pollution levels).

In *demographic studies*, routinely collected information is used to estimate disease rates in populations composed of individuals classified by limited demographic characteristics (e.g., age and sex), allowing for the identification of subgroups at particularly high risk and of changes in the rates over time.

Population-based registries can detect changes in the incidence of the outcomes being registered (e.g., spontaneous abortions, low birth weight, birth defects, neonatal deaths). If placed in selected areas of suspected high risk and "clean" areas of presumed low risk, they may point up differences potentially resulting from environmental causes. Their case materials are a useful resource for mounting case-control studies of suspected environmental hazards (see below).

Analytic studies for formally testing hypotheses and quantifying risks

In the *case-control* design, a series of individuals with an observed effect (the outcome of interest) and a series of unaffected individuals are compared with respect to their previous exposure to the environmental agent of interest. The case-control method is most appropriate for the study of extremely rare outcomes, such as ambiguous genitalia and specific birth defects, and relatively rare outcomes, such as infertility and ectopic pregnancy. It is not useful for the study of very rare exposure unless the study is conducted in a selected setting with sufficiently large numbers of exposed persons (e.g., occupational settings). Case-control studies are not feasible when previous exposures cannot be ascertained.

In *cohort studies*, cohorts of exposed and unexposed individuals are followed for the subsequent occurrence of the outcomes of interest. This design may be the most desirable method for studying fairly common outcomes (e.g., spontaneous abortions), for determining conception rates, and for evaluating subtle indications of reduced fertility, such as variations in interpregnancy interval.

Limitations

All observational studies are subject to certain limitations. Studies classified as hypothesis-generating lack information on potential distorting factors; for this reason, among others, they cannot be used to establish cause-effect relationships. Formal analytic studies do have this capacity if they are valid (i.e., relatively free of biases attributable to selection, measurement, and confounding).

If losses to follow-up are related to outcome status (cohort studies) or if entry into the study is related to exposure status (case-control studies), then *selection bias* will be present. This can occur in industrial settings, for example, if individuals who are exposed to hazardous substances tend to leave the industry and become lost to follow-up because they become ill.

Errors in measurement of exposure or outcome, if unequal between the groups being compared, can lead to overestimation or underestimation of an effect. Equal measurement errors will always lead to attenuation of an effect, and this is a particular problem when exposure or outcome is difficult to measure, as is the case with many environmental exposures and some reproductive outcomes. In cohort studies, there is particular concern that the ascertainment of subsequent outcome be unbiased, while in case-control studies, there is particular concern that the measurement of prior exposure to the agent of interest be unbiased.

Because observational epidemiologic studies deal with nonrandomized populations, a central concern is whether the groups being compared are similar in all relevant characteristics. If they differ in factors related to both the exposure and the outcome, then *confounding bias* will be present. Properly conducted epidemiologic studies will make allowance for all known risk factors of the health outcome of interest, either in the design or in the analysis.

For assessment of the validity of a particular study, detailed information about recruitment and participation of the study population, measurement of the parameters of interest, method of analysis, and efforts to assess potential biases must be available. A single epidemiologic study, even if valid, can seldom by itself rule out chance or bias as the explanation of an observed association. Establishment of causal associations usually requires the accumulation of consistent evidence from valid studies of human populations. That cause precedes effect must also be demonstrated. Belief in a particular hypothesis can be strengthened by evidence from animal studies, by a biologically plausible mechanism, and by a dose-response relationship. In general, the stronger an association, the easier it is to establish a causal association. Conversely, the smaller the effect, the more difficult it is to demonstrate. Failure to detect an effect may simply reflect inadequate sample size or insufficient time for the outcome to become manifest.

This brief review touches on only a few of the potential limitations of observational epidemiologic studies. The assessment of the validity of any particular study requires extensive knowledge of epidemiologic methods and experience with their application.

Possible data sources and useful approaches

Currently few epidemiologic studies attempt to detect human teratogenic and reproductive hazards or to quantify their effects. Furthermore, there is no systematic application of epidemiologic methods for this purpose. It would be desirable to have programs specifically designed to raise suspicions and to test hypotheses. To be effective, these programs must be supported on an ongoing basis.

Before pilot testing any new epidemiologic program, however, the potential usefulness of existing studies and data bases should first be evaluated. Several examples of potentially useful systems are given below.

There are several registries of birth defects in the United States, for example, the Birth Defects Monitoring Program of the Center of Disease Control, which collects information from selected hospitals throughout the country, and the birth defects registries of metropolitan Atlanta and of Nebraska and Florida. The development in selected regions of population-based registries of reproductive health outcomes (including birth defects, ectopic pregnancies, and spontaneous abortions) could point to potential environmental hazards. Even in a particular geographic area, clusters of cases or changes in rates over time could suggest a source of environmental contamination.

Vital statistics have been analyzed from time to time, depending upon the interest of the investigator. For example, infant mortality rates have correlated with chlorination levels of public water supplies in New York (1). In addition, vital statistics have been used as indicators of reduced male fertility in an occupational setting (2, 3). A systematic ongoing analysis of vital statistics data in relation to routinely collected environmental data might be quite useful for raising suspicions about environmental hazards.

Two ongoing surveillance systems based on the case-control approach are currently in operation: one is designed to discover and to evaluate adverse drug effects that are serious enough to warrant hospitalization (4, 5), and the second is directed to the discovery and evaluation of drug teratogenic effects (6). In principle this methodology is applicable to the discovery of environmental agents that are reproductive hazards or teratogens. For the surveillance of occupational exposures, programs could be located in specified areas of the country where occupational exposures to suspected hazards are high. The application of this methodology to the study of nonoccupational environmental exposures is more difficult, in part because individuals may not be aware of what they have been exposed to.

The cohort method has been used to identify several health hazards in occupational cohorts (7, 8). Other cohorts that might be useful are enrollees in health maintenance organizations (HMOs) (see, for example, Ref. 9). However, an HMO data base has the limitation that only outcomes that come to medical attention can be studied. Moreover, no HMO currently has a computerized data base in a form that would be useful for the conduct of epidemiologic studies of environmental exposures. A large investment would be required to build and maintain such a data base.

Pharmacokinetics

Pharmacokinetics can be defined as the quantitative study of the absorption, disposition, metabolism, and elimination of drugs, poisons, and other chemical agents in the body. It is important to evaluate pharmacokinetic variables at different doses and routes of exposure to understand the toxicological significance of exposure.

Pharmacokinetics can be employed for at least two purposes: definition of the concentration levels of the agent in blood or in tissues where the site of action is presumably located, and quantitative description and prediction of the relevant concentration levels, usually with a mathematical model. The first should be routinely done to the extent possible as an aid in interpreting other measurements being made. The second requires much more comprehensive study but has the potential for predictive purposes such as risk assessment.

At this time, there are several basic textbooks of pharmacokinetics: Notari (10) presents an elementary overview, but with many applications; Wagner (11) and Gibaldi and Perrier (12) provide collections of the basic mathematical models and solutions with illustrations of their use. These classical treatments permit organization of pharmacokinetic data, along with some biological interpretations of amounts of an agent in the "central" regions of the body (blood, vital organs) versus "peripheral" regions (other tissues). However, for use of measured levels in specific tissues, models incorporating what is known about quantitative aspects of anatomy and physiology have been found useful (13, 14). Another important feature of this alternative approach is to enable use of known physiological and pharmacological differences between animal species to define some of the critical parameters for quantitative extrapolation to man. A review is given by Dedrick (15), and suggestions for defining similarities between animal species are described by Dedrick and Bischoff (16).

A survey of application of the above pharmacokinetic approaches to some areas of toxicology is given by Gehring et al. (17), and further discussions are in chapters of World Health Organization Environmental Health Criteria (18) and Filov et al. (19). Some specific issues of importance concerning reproductive and teratogenic effects are described by Young and Holson (20).

When pharmacokinetics is applied to the specific area of teratology, the major determinants of the teratogenic agent's reaching and accumulating in the conceptus are the usual aspects of pharmacokinetics in the mother, plus the unique features of transplacental transport, and pharmacokinetics in the conceptus. The maternal pharmacokinetics may be monitored by the blood half-life (20), although it may also be desirable to have more complete details of the disposition into the uterine tissue, as well as the presence of active metabolites and inducible catabolic enzymes, later mobilization of stored agent, and any differences between pharmacokinetics in chronic versus acute exposures. The uptake and disposition into the conceptus may be partially predicted from knowledge of placental membrane transport parameters (models of oxygen and glucose transport may be a useful basis [22]), specific and nonspecific binding, and other features of the developing fetus. It is crucial to determine these effects during the period of major organogenesis.

Few available studies have applied pharmacokinetic principles to specific areas of female or male reproductive organs, especially with reference to formulating models that could be used for predictive purposes. In one of the few, uptake of cancer chemotherapeutic agents into the human uterus has been successfully described by a physiological pharmacokinetic model, which was then used to formulate clinical dosage regimens (22). In another, Lee and Dixon (23) present the results of their innovative study of the pharmacokinetic determinants of uptake into male gonads.

Clearly, much more needs to be done before pharmacokinetics can be routinely utilized as an adjunct in better defining the basis for risk assessment of reproductive and teratogenic toxic effects. However, information obtained using the reasonably well developed methods described in the earlier references should aid in developing methods to resolve some of the issues.

Sexual Behavior

Introduction

Overview. The behavioral aspects of reproduction encompass a broad spectrum of activities including courtship behavior, sexual behavior, parental behavior, and a variety of social activities that subtly influence the probability of reproductive success. The scope of this discussion is limited primarily to sexual behavior for the following reasons.

- This behavioral aspect of reproduction has received the most detailed and extensive attention from clinicians and laboratory investigators.
- Evaluation procedures for sexual behavior of a variety of animal species are well established.
- Choices can be made among several existing standardized procedures currently in use in laboratories around the country.
- Observational methods are simple and direct, and workers at a moderate level of skill can be quickly trained to obtain reliable measurements.
- Many of the testing procedures recommended in the following presentation can provide insight into the probable locus of action of the putative toxicant, and this could not be as easily achieved if the scope of the investigations was extended to include at this time other behavioral aspects of reproduction.

The presentation that follows attempts to establish the impact and causes of sexual dysfunction in humans, to discuss methods of assessing human sexual behavior, and to indicate the difficulties associated with investigations of human sexuality when there are neither controls nor standard norms. The evaluation of sexual behavior patterns in animals are presented as simple tallies of specific motor responses; however, it will be emphasized that many elements of human sexual behavior are unique, having no animal counterpart, thus making uncertain any extrapolation of data on animal sexual behavior to humans.

Definition and scope. The study of sexual behavior encompasses the measurement of normal and abnormal function as well as the identification of the factor(s) responsible for the impairment of sexual behavior. The quantitative measurement of sexual functioning involves the establishment of norms or averages for groups and for the individual and most often focuses on coitus itself. For humans more extensive and varied measures are necessarily employed, which include sexual imagery, sexual fantasy, varieties of overt sexual experience, self concept and gender identity, assessment of interpersonal relationships, choice of sexual object, and level of sexual skill.

For the most part, animal models available today do not provide data that might be required for assessing human sexual functioning and for identifying the factors responsible for imparied expression of sexual behavior. Nevertheless, important advances made in the study of animal sexual behavior provide at least for the initial screening of toxicants that could deleteriously affect human sexual conduct. To identify the factors responsible for normal and impaired sexual behavior, investigators of animal sexual behavior have identified three separate components: sexual attractiveness, sexual initiative, and sexual responsiveness. The current working assumption is that these factors have a much broader generalizability across species than any isolated species-typical behavioral response or activity (e.g., mounting). The evidence and arguments favoring this working assumption have been set forth persuasively by Beach (24), who uses the alternative terminology of attractivity, proceptivity, and receptivity to designate the three factors. Accepted systems of measurement have been worked out for a variety of laboratory animals including the rat and macaque monkeys (25–27).

It should be realized from the outset that manifest sexual behavior reflects the functional integrity of a broad system comprising elements of drive and reward, perception, sensory function, motor performance, the physicochemical actions of gonadal hormones on neural and somatic tissues, and, finally, central nervous system processing and coordinating of the interactions of all of these elements. An efficient screening system utilized for detection of toxic effects should attempt at some stage to distinguish between general motoric disability (ataxia) and impairment of specific sexual reflexes; between general lassitude and loss of specific sexual interest or motivation; and between the impaired production of gonadal hormones and the impaired actions of these hormones. At this time, however, no simple and efficient test of sexual behavior or tests designed to measure sexual attractiveness, initiative, and responsiveness automatically determine whether elements of the broad system are impaired either as a result of general debilitation or selectively and specifically with regard to sexual performance. Currently available tests, while not permitting decisions about the specificity of the effect of a toxic substance, can serve as early warning signals that normal reproductive function has been impaired.

Impact of sexual dysfunction on humans. Data on the incidence of human sexual dysfunction and its spontaneous remission are neither extensive nor very reliable. Certainly the most common clinical problems are primary and secondary anorgasmia in females and *ejaculatio praecox* and erectile impotence in males. These disorders, however, represent extremes of dysfunction that are unacceptable to most humans, and those so affected commonly seek clinical help. Less extreme forms of inadequate sexual response clearly exist and are often tolerated, but only in the sense that professional counseling is not sought. Even though many individuals are reluctant to seek professional help, the importance of sexual behavior and sexual gratification to the overall quality of life and individual well-being is generally recognized. Many individuals are willing to relinquish reproductive capabilities (through vasectomy or other contraceptive means) to enjoy fewer restrictions on sexual activity. Few people, however, will relinquish sexual gratification to gain contraception.

The importance attached to sexual gratification by individuals in our society implies that sexual inadequacy, even when tolerated, may not be without serious consequences. Our monogamous social system depends in a very fundamental way upon a sexual contract between two individuals. Failure to achieve, or even reduction of, sexual satisfaction seriously threatens the interpersonal relationship, as the growing number of marriage counselors recognize. How much of the growing sexual and marital discord is attributable to sociopsychological factors and how much might be attributable to disturbances in the physiological systems underlying sexual performance is not known. The possibility exists, however, for toxic substances in the environment to cause disturbances in sexual performance and thereby contribute to interpersonal discord.

Causes of impaired sexual performance. Sexual dysfunction in human beings is poorly understood. Many sexual disorders are primarily psychogenic and respond well to psychological treatment. Others that are resistant to psychological approaches seem to originate in specific genetic factors, early experience, or a combination of genetic and experiential determinants.

Endocrinopathies, especially abnormalities of the gonadal hormones, have marked influences on the pattern of sexual behavior displayed by animals, and although their influence is less well described for humans, it cannot be said that their role is negligible. For both the male and the female, inadequate gonadal hormone activity commonly results in deficient sexual performance. Subnormal effectiveness of gonadal hormones can be due to (a) deficiencies in production, (b) deficiencies in bioavailability, and (c) deficiencies in target organ sensitivity and/or responsiveness.

Androgen deficiency affects male behavior in two distinct ways. First, during early stages of development (probably before birth in humans) deficiencies in androgen lead to incomplete development of central neural and peripheral somatic structures essential for the expression of masculinity and male behaviors including, but not limited to, male sexual behavior. Second, during adolescence and adulthood, deficiencies in androgen are associated with reduced sexual responsiveness and sexual initiative.

Behavioral disorders associated with excessive androgen have not been identified for the male, although there are recurrent suggestions that excessive amounts during early stages of development lead to permanent androgen insensitivity. In the female, however, excessive androgen during early developmental stages leads to the development of masculine behavioral and somatic characteristics and, in some species, to the suppression or loss of feminine behavior traits. This suppression of feminine traits can include sexual behavior, and female sexual responsiveness can be only reduced. In adulthood, excessive androgen in female humans may lead to measurable somatic virilization (such as hirsutism and clitoromegaly) without any marked effect on psychological and behavioral traits. Increases in sexual initiative and responsiveness may be large enough to be distressing and disruptive to an established interpersonal relationship. In nonhuman primates, androgens have also been implicated in the control of proceptivity (sexual initiative), but not in the control of receptivity. In other mammalian animals, excessive androgen in adulthood may cause a sharp increase in the frequency of malelike mounting activity and aggression with or without concomitant alterations in female sexual behavior. Most of the psychological and somatic changes induced by excessive androgen in adulthood are partially or totally reversible when the hormonal excess is eliminated; however, some of the more dramatic somatic changes (e.g., voice changes and hirsutism) are irreversible.

Estrogen and progestagens play essential but incompletely understood roles in the regulation of female sexual response. These hormones are produced and secreted in much larger amounts by the ovaries than by the testes or the adrenals in normal physiological conditions. Whereas human female sexual behavior does not depend entirely upon the actions of estrogens and progestagens, its expression is greatly facilitated by their actions on both central and peripheral neural and somatic tissues. In animals, especially the common laboratory forms, the ovarian hormones are much more essential to the expression of female sexual behavior than in human beings. Generally, the effective estrogen is estradiol and the effective progestagen is progesterone. These two steroid hormones act synergistically in the induction of both proceptivity and receptivity in rats, mice, hamsters, and guinea pigs. The two hormones may also act antagonistically, however, and which relationship obtains depends upon whether or not the estrogen has been free to act for a specifiable period of time without any concurrent actions of a progestagen. The synergistic relationship depends upon the sequential action of an estrogen followed (usually 36 to 48 hours later) by the action of a progestagen. An antagonistic relationship will be evidenced whenever a progestagen and an estrogen are both present throughout the period of observation or study.

Excessive estrogenization acts to lengthen the period or duration of receptivity and proceptivity. An established norm of eight hours for the duration of receptivity in a colony of rats can be extended to 12 or 14 hours by excessive estrogenization. In extreme cases, excessive estrogenization can extend receptivity indefinitely.

Excessive progesterone has no measurable effect if the period of stimulation is brief. If the period of excessive stimulation is protracted, however, receptivity and proceptivity can be indefinitely suppressed or inhibited. The antagonistic effect of progestagens is transitory and reversible when these hormones are brought back to physiologic concentrations.

It is difficult but not impossible to distinguish between the antagonistic effects of excessive progestagen and a deficiency in estrogenization. A deficiency of estrogen, like excessive progestagenization, has the primary characteristic of weak or absent female sexual response. A distinction between the two possible causes of impaired sexual response can be made by institution of appropriate experimental hormone administration to ovariectomized females.

Excessive estrogen or progestagen during early periods of development can produce permanent deficiencies in female sexual response in a variety of laboratory animals. Comparable data for human beings do not exist.

Sequelae of estrogen and/or progestagen excess or deficiency in males have not been well worked out. Supraphysiologic levels of estrogen have been administered to human males in cases of prostatic cancer. Sexual drive and erectile potency sometimes decline in these cases, presumably because the estrogens block the release by the pituitary of testis-stimulating hormone, and an androgen deficiency results. Similar effects could be obtained in some laboratory animals (the guinea pig), but not in others (the rat, in which excessive estrogens mimic androgens in the potentiation of male sexual activity).

The effects of hormone excess and deficiency could occur when chemical substances mimic or antagonize physiological actions of the relevant gonadal hormone. Other chemical agents could enhance the degradation of steroidal hormones in the liver or kidneys and thereby reduce or limit their effectiveness. Still other chemicals could either act on the hypothalamic-pituitary system to modify the release of trophic substances essential for the normal production of the gonadal hormones or act directly upon the glandular tissues responsible for their production.

Many factors, aside from alteration of or interference with hormonal support, can act to impair sexual behavior. These factors are difficult to assess in standard laboratory tests, either because no suitable animal model can be found or because appropriate assessment would involve procedures too elaborate and costly for routinization. Although testing for alteration or interference with the hormonal support of sexual behavior assesses only a limited aspect of requirements for human sexual adequacy, it has the advantage that the information gained is reliable, quantitative, and amenable to use in estimating the risk to human sexuality posed by specific chemical substances.

Qualitative evaluation of risk potential

Interspecies comparisons. Requirements for genetic variability in the test model animal that approximates that encountered in the human population have already been discussed in the section titled "Interspecies Comparisons" in Chapter 4. Highly inbred strains ought to be generally avoided unless several are used to determine the range of sensitivity to the test substance.

Known and suspected differences among species in the manner in which gonadal hormones regulate sexual behavior mandate the use of more than one species. For example, the major androgen produced by the testis is testosterone in most mammals. This hormone is metabolized within somatic and neural cells to a variety of other steroids including estradiol and dihydrotestosterone. In some species, like the rat, the estradiol derived from bioconversion of testosterone is a potent stimulator of male sexual behavior in the adult and a potent masculinizer of the developing brain in the fetus and neonate. In contrast, in the guinea pig this estrogen metabolite of testosterone is without any measurable stimulating effect on male sexual behavior when it is given to castrated adults. The view is widely held that the display of male sexual behavior depends upon the intracellular conversion of testosterone to estradiol in the normal male rat, whereas testosterone acts either directly or via conversion to dihydrotestosterone on the neural tissues mediating male sexual behavior in the guinea pig.

The "rat model" for cellular utilization of testosterone (by conversion to an estrogen) is valid for hamsters and some but not all inbred strains of mice. The "guinea pig model" is valid, based on very limited data, for the rhesus monkey and also for humans.

This species difference is important because erroneous conclusions are possible if testing is limited to a single species. Any putative toxicant that blocks intracellular aromatizing enzymes needed for bioconversion of testosterone to estrogen would impair adult male rat sexual behavior, but the same compound would not likely have an effect on sexual behavior of male guinea pigs, rhesus monkeys, or humans.

Other species differences, too numerous to detail here, include differences in the role of specific neural structures, in the contribution of specific neurotransmitters, in the amount and kind of carrier protein that is present in the bloodstream and binds and transports the steroid hormones, in the chemical structure of the pituitary trophic hormones, and certainly in the form and normal frequency of sexual expression. All of these species differences argue for the use of more than one species in screening for toxicity as well as for judicious choices when only a few are to be used. In short, the choice of a species to use as a model animal places profound and subtle limits on evaluating the toxic consequences of any chemical agent, and these limits have to be reckoned with.

Certain relatively simple and easy-to-conduct tests could serve as a preliminary screen to indicate the degree of likelihood of an agent's affecting either the early sexual differentiation or adult expression of sexual behavior. Based on the assumption that chemical substances that pass the placenta and gain access to the fetal tissues are more likely to affect early development than those that fail to pass through the placenta, a relatively simple and efficient study of the distribution of the radioactively labeled chemical substance could be carried out. It is also reasonable to use radiolabeled material in adult animals to determine whether the substance crosses the blood-brain barrier and has the potential of acting directly on nervous tissue. These simple tests, of course, are not specific indicators that sexual behavior would be altered by the putative toxicant. Positive findings from these tests would merely serve the purpose of indicating increased likelihood.

Other considerations. It is reasonable to assume that a wide variety of other factors are important in facilitating extrapolation of animal tests of a toxicant to the estimation of risk to humans. These include dosages of putative toxicant used, route of administration, duration and frequency of exposure, species thresholds and sensitivities to the chemical substance, and specific pharmacokinetics and pharmacodynamics of the test compound. Whenever information exists for humans on any of these factors, either for the specific test compound or a closely related substance, an effort should be made to select an animal model that most closely parallels the human to increase the applicability of the animal test results.

Despite the advantages of objectivity, ease of administration, reliability, and wealth of background information, tests of animal sexual behavior in the present context have severe limitations. First and foremost is the high degree of uncertainty that results of animal tests could be extrapolated to human sexual behavior. It is likely that extrapolation would be good if a putative toxicant completely blocked the neurological actions of the sex hormones (especially the androgens), since hormonal support for sexual behavior and for the fetal differentiation of sexual and/or sex-related behavior is a factor common to both animals and humans. However, humans and animals differ greatly in the numbers and kinds of nonhormonal factors influencing the expression of sexual behavior. Accordingly, when a putative toxicant acts only on one or a subset of nonhormonal factors, there is a strong likelihood that animal test results will not correspond to effects (or lack thereof) on human sexual expression.

A second area of concern is the nearly total lack of background information on effects of known toxicants on sexual behavior in either animals or humans. This deficiency thwarts any present attempt at formulation of procedures for quantitative risk assessment based on findings from animal tests. This situation can be remedied only by providing encouragement of the appropriate research on animal models as well as intensive studies of humans exposed to known toxic agents.

Animal studies

Evaluation of sexual behavior in adulthood. Observations of sexual behavior in adult animals can be made by easily trained nonexperts. Useful assessments of the status of sexual behavior can be made from simple tallies of the frequency of occurrence of specific motor responses and the latent period from the beginning of a standardized test to the occurrence of the specific response. These are the operational measures of initiation, attractiveness, and responsiveness.

The procedures described in this section are designed to permit reliable, sensitive analyses of the effects of potentially active chemical substances on male sexual behavior. A considerable body of knowledge gathered in the last 60 years reveals that the sexual patterns of rats and guinea pigs can provide such data. Further, the existence of extensive data bases on these two species provides the possibility of a preliminary indication of mechanism of action underlying observed treatment effects, since determinants of various aspects of these complex patterns have received much study. Should more extensive and expensive testing of a chemical be indicated, dogs, nonhuman primates, or other species may be appropriate. Methods described below can be adapted to such species using behavioral testing procedures described by Dewsbury (28) and in the references therein.

In all tests, one sex should be treated so that treatment effects may be detected uncomplicated by effects of the agent's acting on the opposite sex. Where the probability of a treatment effect is quite low, it may be more economical to combine procedures for male and female treatments into a single protocol. However, the risk that three rather than two studies may be required if such procedures are used should be recognized.

Assessing sexual behavioral patterns of males. Effects of various toxicants can be evaluated as they alter the normal complex behavioral patterns of male rats and guinea pigs. There are some considerable advantages to toxicological inquiry in studying the behavior of male animals that have been castrated and given physiological hormone replacement and exogenous testosterone. This procedure obviates the possibility that impaired sexual performance might be due to toxic insult to the hypothalamic-pituitary-testicular axis or to the testis itself. However, inasmuch as castration and replacement therapy are complicated techniques in themselves, testing the intact animal should serve as an adequate preliminary screen.

In studying the normal copulatory behavior of laboratory rats, three classes of events are generally distinguished – mounts, intromissions, and ejaculations. With the first the male mounts the female from behind, displays shallow pelvic thrusting, but neither gains vaginal penetration nor displays the stereotyped pattern of dismounting. Intromissions begin similarly, but the male achieves a single deep thrust and dismounts in a vigorous and stereotypical pattern. Ejaculations occur only after several intromissions and are characterized by an intravaginal thrust that is longer and deeper than that of intromissions. Sperm are transferred only on ejaculations. The male mounts the female during mounts, intromissions, and ejaculations, but the three classes of events are distinguished as just indicated. During pair mating copulatory events occur in "ejaculatory series," with each series terminated by an ejaculation and separated from a resumption of copulation by a postejaculatory refractory period. In standard testing cages, males normally display a mean of approximately seven ejaculatory series before attaining an arbitrary, but standard, satiety criterion of 30 minutes with no intromissions or ejaculations.

Standard measures of male copulatory behavior include mount latency (ML), time from start of a test to the first mount or intromission; intromission latency (IL), time from the start of a test to the first intromission; ejaculation latency (EL), time from the first intromission of a series to its terminal ejaculation; intromission frequency (IF), number of intromissions in a series; mount frequency (MF), number of mounts in a series; mean interintromission interval (MIII), mean interval separating the intromissions within a series; and postejaculatory interval (PEI), time from ejaculation to the next intromission. Male receptivity may be quantified by dividing the number of male chase and follow-bouts by the total number of female approaches (29).

Because various of these measures can be affected selectively, specifically, and in combination, an accurate interpretation of a treatment effect requires a full complement of these measures. As an example, suppose a treatment interfered with the process of penile erection. Males with such problems often mount females at rates much higher than normal as they repeatedly attempt to effect intromission. Without a full complement of measures, such an effect might be mistaken for an *increase* in libido rather than as a deficit. Similarly, a treatment that alters MIII may secondarily affect IF. In addition, with a full set of measures, one can evaluate the control group in relation to animals used in previous studies (see references below) to ensure that it is providing an appropriate baseline for comparison.

Full descriptions of copulatory behavior in male rats can be obtained in Beach and Jordan (30), Dewsbury (27), Larsson (31), and Sachs and Barfield (32).

Copulatory behavior in male guinea pigs differs from that in male rats in several important respects. First, whereas rats display but a single intravaginal thrust on each mount with intromission, guinea pigs display repetitive thrusts on a single insertion. Second, whereas male rats rarely, if ever, ejaculate on the first mount with intromission, such occurrences are more frequent in guinea pigs. Third, although male rats normally display several ejaculations per test session, the occurrence of the first ejaculation generally effectively terminates copulatory activity in guinea pigs. In other respects, the copulatory patterns of male guinea pigs are quite similar to those of male rats. Similar measures can be used.

Descriptions of copulatory behavior in guinea pigs can be found in Young (33) and Young and Grunt (34). Various measures of preliminary aspects of courtship and mating described in these papers may be useful.

Test of copulatory behavior should be conducted during the dark phase of the diurnal cycle. By testing during the second half of the dark phase, behavior generally is more reliable, quicker, and less variable – making for a more efficient and sensitive test (35). Tests should be conducted at approximately the same time on each day.

Males and females should be familiar with the testing arenas via introduction several times on days before test days.

In tests for male behavior, males are generally placed in the arenas for five to ten minutes, after which the female is introduced, effectively beginning the test. Tests may be terminated and scored as negative if there is no copulatory activity within a predetermined time (e.g., 15 minutes).

Tests of guinea pigs should be terminated at the occurrence of ejaculation. Those of rats should be continued for two or three ejaculatory series. Such tests may require an average of 45 minutes in rats. It may be feasible to test several pairs of rats simultaneously in cages close to each other, if the only behavioral patterns to be scored are those discussed above.

For reasons of reliability and predictability, it is recommended that female mating partners be brought into behavioral estrus with exogenous hormones. Female guinea pigs must first be ovariectomized; this may or may not be done with female rats. For either intact female rats or spayed guinea pigs, good results can be obtained with an intramuscular injection of 0.1 mg of estradiol benzoate three days before testing and 1 mg of progesterone approximately six hours before testing. Somewhat lower doses can also be used. Females should be placed briefly with a vigorous, nonexperimental "indicator" male immediately before testing to ensure that the injection regimen has been effective in inducing receptivity. A single female rat in estrus can be used to evaluate sexual performance of at least three males. A single female guinea pig should not be used for more than two males.

There are many factors, both quite specific and highly nonspecific, that can alter copulatory behavior. If there are gross increases or decreases in body weight or activity levels, changes in sexual behavior are probably secondary to more general effects. If body weight and general activity is near normal and copulatory behavior is altered, however, greater specificity of action probably is indicated. Some indication of the nature and degree of effect can be determined by considering the constellation of measures altered, the magnitude of effect, and reversibility. By comparing these changes to those described in the literature as resulting from other treatment, some preliminary indication as to probable mechanism of action can be gained. Any alteration requires some further analysis. Such subsequent studies may be directed at analyzing neural, endocrine, and other systems to determine whether or not the effect seems appreciable and likely to affect humans.

Assessing sexual behavior patterns of females. A substantial and useful background of behavioral data exists for both rats and guinea pigs from a number of inbred strains as well as genetically heterogenous stocks. It is possible to evaluate proceptivity, receptivity, and attractiveness in female rats in a single test paradigm with a stud male and to evaluate receptivity in the female guinea pig.

The intact and normally functioning female rat displays a period of estrus ("heat") that lasts from 6 to 11 hours about every 4 to 5 days. As long as the female is not mated, estrus recurs regularly. Recurrent estrus also occurs in the unmated female guinea pig, but the interval between receptive episodes lasts 14 to 17 days. In both species, sexual behavior depends upon appropriate ovarian secretion of estradiol and progesterone. When the ovaries are removed, sexual behavior is no longer displayed. Proceptive and receptive behaviors are displayed in close temporal proximity and have similar hormonal requirements (36).

The behavioral response indicative of normal receptivity is the lordosis posture assumed by the female during mounting by a male partner. Degree of receptivity is estimated in a quantitative fashion by dividing the number of lordosis responses displayed by the female by the number of times she is mounted by her male partner in a standardized test. This derived measure is called the *receptivity quotient* or, alternatively, the *lordosis quotient*. The measure is more useful in the rat than in the guinea pig, because male rats are normally multiple mounters, whereas male guinea pigs often mount only once during a mating test. For the female guinea pig, therefore, an alternative procedure for quantifying receptivity is often used. The procedure, described fully elsewhere (37, 38), involves manual stimulation of the animal's rump and perineum by the human observer and measurement of the degree or duration of the lordosis response to such stimulation. During mating with a stud male, female attractiveness may be quantified by measuring the latency between introduction of the female and a male approach, follow, and mount (29).

Proceptivity can be measured quantitatively in the female rat by recording the frequency and timing of displays of a variety of motor patterns including female solicitations and approaches to the male partner, darting, hopping, and ear vibration. These proceptive patterns generally are displayed just prior to the occurrence of male mounting responses, but they may occur at any time when the male is quiet or inactive.

Full descriptions of female rodent sexual behavior can be found in Diakow and Dewsbury (39); McClintock and Adler (25); McClintock, Ansiko, and Adler (40); and Madlafousek and Hlinak (26).

Evaluation of sexual responses in the intact female requires constant and frequent monitoring of individual animals. This is essential because the occurrence of the behavior is restricted to a short and specific period of the ovarian cycle. The behavior normally is expressed only during the time the follicle is undergoing its final preovulatory swelling. The ovarian cycle is usually monitored by taking daily vaginal smears for cytological evaluation, and sexual behavior usually is displayed during the proestrous smear or the transition between proestrous and estrous smears.

The procedure of monitoring the ovarian cycle by daily vaginal smears is cumbersome, time-consuming, and not very precise with respect to the assessment of sexual behavior. When individual females are tested for receptivity and proceptivity at an arbitrary time relative to a particular vaginal cytology, some may be at the beginning of the period of receptivity, some in the middle, and some near the end. Others may not yet have reached the receptive stage, and for those in various segments of the period the quality of receptive behavior will vary accordingly. Furthermore, in the intact female, impairment or absence of sexual response could be due to impairment of pituitary gonadotrophic activity, disordered ovarian production of steroids, or impairment of the response of relevant neural centers to the gonadal hormones.

Undesirable variability as well as uncertainty about the cause of impaired sexual response can be reduced by assessing sexual behavior in spayed females suitably treated with injections of estradiol and progesterone. Usually females are brought into good states of receptivity by a single subcutaneous injection of estradiol benzoate followed 48 hours later with an injection of progesterone. All animals to be tested can then be evaluated at an exact time (usually six hours) after the progesterone injection.

The artificial induction of sexual responses has to be done with precision and with concern for hormonal stimulation that closely approximates the normal physiological pattern. Administration of excessive amounts of estrogen and progesterone could mask or override subtle derangements induced by a toxic substance. If spayed animals are used for assessment of sexual behavior, great care must be exercised to ensure that physiological doses of estrogen and progestagen are administered. Reference to the literature on experimental analysis of female rodent sexual behavior will not be helpful as a guide to proper hormone treatment, since suprathreshold dosage regimens are usually used, and these are not appropriate for screening toxicants. In any attempt to identify damaging actions of a putative toxicant, the investigator should be cautious about exceeding 1 μg of estradiol benzoate and 0.1 mg of progesterone per adult animal. The best general rule to follow is to conduct an initial parametric study on the specific breed or strain to be used and to determine the minimum hormonal requirements for induction of estrous behaviors in a specified percent of the population.

Assessment of human sexual behavior: surveillance and epidemiological studies

Preliminary comments. Direct assessment of human behavior is essential for evaluating the behavioral effects of environmental toxicants. The extrapolation of animal studies to human behavior is limited for a variety of reasons. (a) Many aspects of human sexuality and reproductive behavior are unique and have no obvious animal counterpart (41, 42). (b) While compounds such as steroids do affect sexual motivation in both animals and humans, their behavioral manifestations in humans are often quite different from their manifestations in animals. (c) Human behavior may be disrupted at lower toxicant levels than would be expected from animal studies. (d) The exposure of the general population, but especially workers, to the compound may be greater in fact than originally estimated (also see "Other Considerations").

We present several different methods for assessing the effect of a toxicant on human sexual behavior. Because direct controls may not be possible for practical or ethical reasons, each method has its own weakness. Therefore, we have proposed a variety of methods and suggest that they be used concurrently if at all possible. This extra effort would be justified particularly when the potential benefits of a compound are high, but also when animal toxicological screening or analysis of the compound's structure indicates that the potential risk to human behavior may also be high. In any case, the behavioral assessment procedures for humans need not be cumbersome and can be incorporated in any procedure or physical exam designed to monitor the effects of a putative toxicant on reproductive function.

Behavioral surveillance of humans potentially exposed to a reproductive toxicant. Ideally, new compounds would be released and used at first on a limited basis. Then, changes in sexual satisfaction and function could be assessed prospectively with adequate controls. The sexual experience of the exposed group, perhaps production workers who would be exposed to higher concentrations, could be compared with a matched group of similar workers in an area or plant where the compound was not yet in use. This comparison should be made between two groups of workers in the same plant or location. If this is not possible, the two groups should be matched for factors known to correlate with sexual attitudes and behavior such as socioeconomic status, cohort, ethnicity, religion, and environmental factors. (Industry should use an epidemiologic consultant to determine the matching criteria, sample size, and duration of surveillance appropriate for the amount of natural variance in the proposed measures of sexual behavior.)

If limited release is not warranted ethically or practically and a general release occurs, it would be necessary to monitor sexual satisfaction and the incidence of dysfunction before as well as after the compound is released. The large population variability in normal sexual behavior may make this procedure more sensitive than a comparison between groups. Furthermore, as the behavior of each person is compared to his own normal pattern, it may be possible to identify a subpopulation of particularly sensitive individuals.

These control procedures are essential for evaluating the effect of a toxicant on human sexual behavior, because standard norms are not currently available as a basis for comparison as they are for such physical variables as sperm count or menstrual cycle length. As the number of controlled studies increases, it may be possible to use the data from control groups to develop normative statistics for future evaluations.

The frequency of sexual intercourse is not a good indicator of sexual satisfaction by itself; it is also necessary to evaluate sexual arousal, initiation, and changes in erotic imagery and to identify specific sources of sexual dysfunction. For example, there was little agreement about the nature of changes in women's sexual motivation over the menstrual cycle until studies focused on behavior of the woman herself and her sexual initiation and fantasy rather than on the frequency of intercourse (43, 44).

Either an interview or a short questionnaire can assess sexual satisfaction and function. It is important that the interviewer be trained in interview techniques for sexual counseling. Short courses are currently available for medical and lay personnel in most academic medical centers (Marriage Counseling Center of the University of Pennsylvania has a list). Alternatively, there are a variety of short questionnaires that correlate well with such physiological measures of sexual arousability as penile tumescence and vaginal lubrication (45, 46) and that generate a similar profile whether completion of the questionnaire is mandatory or voluntary (47, 48).

Another approach to the assessment problem is based on epidemiological data. The incidence of cases involving sexual dysfunction reported to such institutions as mental health clinics, local physicians, or plant infirmaries can be recorded and used as a normative data base. This baseline could be compared with the frequency of reported cases following the release of a new compound. Again, an epidemiologic consultant should determine whether the sample from available institutions would be large enough to detect a toxic effect.

Evaluation of human sexual behavior following exposure to a known toxicant. Many compounds have been established as physiological toxicants but have not yet been assessed for behavioral effects in humans. Estrogenic compounds such as DES and DDT may affect the sexuality of women, while organopesticides that are neurotoxins, such as carbaryl, an acetylcholine esterase inhibitor, may affect male erectile function.

If a population has been exposed to such compounds or is suspected of being at reproductive risk, behavioral assessment can be made at the time that a physiological assessment is being made. The same personnel could do this, provided that they have been trained in interview techniques. Behavioral assessment under these *ex post facto* conditions is particularly difficult because knowledge of exposure to a toxicant can distort the retrospective assessment of sexual satisfaction and behavior. Therefore, trained personnel, an evaluation immediately after the exposure, and established norms would each help to reduce this bias. In any event, an unexposed control population should be evaluated using the identical retrospective procedures and matched to the target population for such variables as socioeconomic status, ethnic group, and local environment.

Risk assessment. If any significant alterations are found, exposure to the toxicant should be discontinued to assess the reversibility of the effects. Furthermore, the mechanism of action will need to be identified to evaluate a risk/benefit ratio. For example, it is possible that erectile function could be impaired through a direct impairment of cholinergic mechanisms or indirectly through an increase in depression or sense of fatigue (49). Nocturnal penile tumescence would aid in a differential diagnosis, as erectile function during sleep is not impaired by psychogenic factors. Human sexuality is particularly sensitive to disruption by many environmental and psychological factors that are not specifically sexual themselves; most instances of sexual dysfunction encountered in the clinic are not the result of a direct organic cause. Therefore, the mechanisms of any impairment of sexual performance or satisfaction will have to be determined before a risk/benefit ratio can be assessed.

Priority areas for future research

Few experimental studies have been made of the effects of toxic substances on the sexual behavior of laboratory animals. A few recent references (50–61) are included in the listing at the end of this chapter, but they deal primarily with effects of drugs like cannabis, alcohol, and morphine. A search of the literature between 1978 and 1980 revealed only two references dealing with other agents, one on cyanogenic substances (62) and the other on lindane (63). In addition, no systematic evaluations of sexual behavior have been conducted on humans known to have been exposed to toxic substances either in adulthood or prenatally. This unfortunate circumstance, that parallel studies have not been carried out on intentionally exposed animal subjects and on accidentally exposed human beings, severely limits the capability to formulate either qualitative or quantitative risk assessments for sexual functioning.

Basic research on human sexual behavior should be strongly encouraged at this time so that appropriate demographic norms and standards can be established. Adequate information on these matters has not been developed despite the pioneering efforts of Kinsey in the late forties. In addition, changes in concepts, data gathering techniques, and attitudes require modernization of the data base. As pointed out in earlier sections of this discussion, neither measurement of number of offspring produced, frequency of coitus, or even frequency of orgasm are adequate as indicators of human sexual functioning. There is a strong need to develop epidemiological studies of human sexual behavior in its broadest scope and in terms most meaningful to human welfare and to the stability of interpersonal relationships.

The scope of investigations of animal sexual behavior should be broadened. Efforts to establish models permitting better measurement of sexual attractiveness, sexual motivation or desire, and even sexual gratification should be encouraged. Moreover, sound parametric data on the effects of known environmental toxicants ought to be vigorously pursued. These studies could be carried out profitably at this time even with the limited number of behavioral measures currently available, and there should be strong support for such studies on a variety of species. The limitation of data bases, no matter how extensive, to rats and guinea pigs poses a serious obstacle to flexible choice of alternative models that may in fact be more appropriate to human problems.

Finally, even from the relatively limited standpoint of sexual behavior, more information is needed on how different classes of chemical substances interact with central neural tissues on the cellular levels. Information of this sort is fundamental not only to interpretation of toxicant effects on behavior, but also to sound hypothesis formulation and to development of a framework that would permit prediction of the likely biological effects of a putative toxicant.

REFERENCES

1. Rausch, L., Klein, J., Shaffer, S. Q. and Stein, Z.: Effect of chlorination of drinking water on perinatal mortality in New York villages, 1968-1977. Abstract. Am. J. Epidemiol. 112: 439, 1980.
2. Levine, R. J., Symons, M. J., Balogh, S. A., Arndt, D. M., Kaswandik, N. T. and Gentile, J. W.: A method for monitoring the fertility of workers: I. Method and pilot studies. J. Occup. Med. 22: 781–791, 1980.
3. Levine, R. J., Symons, M. J., Balogh, S. A., Milby, T. H. and Whorton, M. D.: A method for monitoring the fertility of workers. II. Validation of the method among workers exposed to dibromochloropropane. J. Occup. Med. 23: 183–188, 1981.
4. Slone, D., Shapiro, S. and Miettinen, O. S.: Case-control surveillance of serious illnesses attributable to ambulatory drug use. In: Epidemiological Evaluation of Drugs, F. Colombo, S. Shapiro, D. Slone, and G. Tognoni, Eds., Elsevier North-Holland Biomedical Press: Amsterdam; pp. 59–82, 1977.
5. Rosenberg, L., Shapiro, S., Slone, D., Kaufman, D. W., Miettinen, O. S. and Stolley, P. D.: Thiazides and acute cholecystits. N. Engl. J. Med. 303: 546–548, 1980.
6. Mitchell, A. A., Rosenberg, L., Shapiro, S. and Slone, D.: Birth defects related to use of Bendictine in pregnancy. I. Oral clefts and cardiac defects. J. Am. Med. Assoc. 245: 2311–2314, 1981.
7. Selikoff, I. J., Churg, J. and Hammond, E. C.: Relation between exposure to asbestos and mesothelioma. New Engl. J. Med. 272: 560–565, 1965.
8. Lee, A. M. and Fraumeni, J. F., Jr.: Arsenic and respiratory cancer in man: An occupational study. J. Natl. Cancer Inst. 42: 1045–1052, 1969.
9. Harlap, S., Shiono, P., Ramcharan, S., Berendes, H. and Pellegrin, F.: A prospective study of spontaneous fetal losses after induced abortions. New Engl. J. Med. 301: 677–681, 1979.
10. Notari, R. E.: Biopharmaceutics and Pharmacokinetics: An Introduction. 3rd Edition, Marcel Dekker: New York; 329 pp., 1980.
11. Wagner, J. G.: Biopharmaceutics and relevant pharmacokinetics. Drug Intelligence Publications: Washington, D.C.; 375 pp., 1971.
12. Gibaldi, M. and Perrier, D.: Pharmacokinetics. Marcel Dekker: New York; 325 pp., 1975.
13. Bischoff, K. B.: Some fundamental considerations of the application of pharmacokinetics to cancer chemotherapy. Cancer Chemother. Rep. Part 1 59: 777–796, 1975.
14. Himmelstein, K. J. and Lutz, R. J.: A review of the applications of physiologically based pharmacokinetic modeling. J. Pharmacokinet. Biopharm. 7: 127–145, 1979.
15. Dedrick, R. L.: Animal scale-up. J. Pharmacokinet. Biopharm. 1: 435–461, 1973.
16. Dedrick, R. L. and Bischoff, K. B.: Species similarities in pharmacokinetics. Fed. Proc. 39: 54–59, 1980.

17. Gehring, P. J., Watanabe, P. G. and Blau, G. E.: Pharmacokinetic studies in evaluation of the toxicological and environmental hazard of chemicals: In: Advances in Modern Toxicology: New Concepts in Safety Evaluation, M. Mehlman, H. Blumenthal, and R. Shapiro, Eds., Hemisphere Publishing Corporation: New York; pp. 195–290, 1976.

18. World Health Organization. Chemobiokinetics and metabolism. In: Environmental Health Criteria 6. Principles and Methods for Evaluating the Toxicity of Chemicals. Geneva; pp. 116–177, 1978.

19. Filov, V. A., Golubev, A. A., Liublina, E. I. and Tolokontsev, N. A.: Quantitative Toxicology. John Wiley: New York; 462 pp., 1980.

20. Young, J. F. and Holson, J. F.: Utility of pharmacokinetics in designing toxicological protocols and improving interspecies extrapolation. J. Environ. Pathol. Toxicol. 2: 169–186, 1978.

21. Souchay, A. M., Rice, P. C., Rourke, J. E. and Nesbitt, R. E. L.: Glucose metabolism and transfer in the human placenta. In: Chemical Engineering in Medicine, D. D. Reneau, Ed., Plenum Press: New York; pp. 172–199, 1973.

22. Dedrick, R. L., Meyers, C. E., Bungay, P. M. and De Vita, V. T.: Pharmacokinetic rationale for peritoneal drug administration in the treatment of ovarian cancer. Cancer Treatment Rep. 62: 1–11, 1978.

23. Lee, I. P. and Dixon, R. L.: Factors influencing reproduction and genetic toxic effects on male gonads. Environ. Health Perspect. 24: 117–127, 1978.

24. Beach, F. A.: Animal models for human sexuality. In: CIBA Foundation Symposium, No. 62. Sex, Hormones, and Behavior, Mar. 14–16, 1978, London, England, Elsevier-North Holland: New York; pp. 133–144, 1979.

25. McClintock, M. K. and Adler, N. T.: The role of the female during copulation in wild and domestic rats (*Rattus norvegicus*). Behavior 67: 67–96, 1978.

26. Madlafousek, J. and Hlinak, Z.: Sexual behavior of the female laboratory rat: inventory, patterning, and measurement. Behavior 63: 129–174, 1977.

27. Dewsbury, D. A.: A quantitative description of the behavior of rats during copulation. Behaviour 29: 154–178, 1967.

28. Dewsbury, D. A.: Description of sexual behavior in research on hormone-behavior interactions. In: Endocrine Control of Sexual Behavior, C. Beyer, Ed., New York: Raven Press, pp. 3–32, 1979.

29. McClintock, M. K., Toner, J. P., Adler, N. T. and Anisko, J. J.: Postejaculatory quiescence in female and male rats: consequences for sperm transport during group mating. J. Comp. Physiol. Psychol.; in press, 1982.

30. Beach, F. A. and Jordan, L.: Sexual exhaustion and recovery in the male rat. Q. J. Exp. Psychol. 8: 121–133, 1956.

31. Larsson, K.: Conditioning and Sexual Behavior in the Male Albino Rat. Stockholm: Almquist & Wiksell; 1956.

32. Sachs, B. D. and Barfield, R.: Functional analysis of masculine copulatory behavior in the rat. Adv. Study Behav. 7: 91–154, 1976.

33. Young, W. C.: Psychobiology of sexual behavior in the guinea pig. Adv. Study Behav. 2: 1–110, 1969.

34. Young, W. C. and Grunt, J. A.: The pattern and measurement of sexual behavior in the male guinea pig. J. Comp. Physiol. Psychol. 44: 492–500, 1951.

35. Dewsbury, D. A.: Copulatory behavior of rats: Variations within the dark phase of the diurnal cycle. Commun. Behav. Biol. 1: 373–377, 1968.

36. Fadem, B. H., Barfield, R. J. and Whalen, R. E.: Dose-response and time-response relationships between progesterone and the display of patterns of receptive and proceptive behavior in the female rat. Horm. Behav. 13: 40–48, 1979.

37. Goy, R. W. and Young, W. C.: Strain differences in the behavioral responses of female guinea pigs to alpha-estradiol benzoate and progesterone. Behaviour 10: 340–354, 1957.

38. Walker, W. A. and Feder, H. H.: The comparative potency of various steroids to complete the priming process for lordosis in guinea pigs. Horm. Behav. 12: 299–308, 1979.

39. Diakow, C. and Dewsbury, D. A.: A compuative description of the mating behavior of female rodents. Anim. Behav. 26: 1091–1097, 1978.

40. McClintock, M. K., Anisko, J. J. and Adler, N. T.: Group mating among Norway rats. I. Sex differences in the pattern and neuroendocrine consequences of copulation. Anim. Behav.; in press, 1982.

41. Beach, F. A.: Cross-species comparisons and the human heritage. In: Human Sexuality in Four Perspectives, F. A. Beach, Ed., Johns Hopkins University Press: Baltimore; pp., 296–316, 1977.

42. Beach, F. A.: Animal models and psychological inference. In: Human Sexuality: A Developmental and Comparative Approach, H. A. Katchadourian, Ed., University of California Press: Berkeley; pp. 98–112, 1979.

43. Adams, D., Gold, A. and Burt A.: Rise in female-initiated sexual activity at ovulation and its suppression by oral contraceptives. N. Engl. J. Med. 299: 1145–1150, 1978.

44. Persky, H., Lief, H., Strauss, D., Miller, W. and O'Brien, C. D.: Plasma testosterone level and sexual behavior of couples. Arch. Sex. Behav. 7: 157–173, 1978.

45. Byrne, D. and Scheffield, J.: Response to sexually arousing stimuli as a function of repressing and sensitizing defenses. J. Abnorm. Psychol. 70: 114–118, 1965.

46. Byrne, D., Fisher, J. D., Lamberth, J. and Mitchell, H. E.: Evaluations of erotica: Facts or feelings. J. Pers. Soc. Psychol. 29: 111–116, 1974.

47. Kaats, A. and Davis, K.: Effects of volunteer biases in studies of sexual behavior and attitudes. J. Sex. Res. 7: 219–227, 1971.

48. Bauman, K.: Volunteer bias in a study of sexual knowledge, attitudes, and behavior. J. Marr. Fam. 35: 27–31, 1975.

49. Kaplan, H. S.: The New Sex Therapy, Brunner/Mazel: New York; 544 pp., 1974.

50. Abel, E. L.: A review of alcohol's effects on sex and reproduction. Drug Alcohol Depend. 5: 321–332, 1980.
51. Barnes, T. R., Bamber, R. W. and Watson, J. P.: Psychotropic drugs and sexual behaviour. Br. J. Hosp. Med. 21: 594–600, 1979.
52. Clemens, L. G., Popham, T. V. and Ruppert, P. H.: Neonatal treatment of hamsters with barbiturate alters adult sexual behavior. Dev. Psychobiol. 12: 49–59, 1979.
53. Dalterio, S. L.: Perinatal or adult exposure to cannabinoids alters male reproductive functions in mice. Pharmacol. Biochem. Behav. 12: 143–153, 1980.
54. Dalterio, S. and Bartke, A.: Perinatal exposure to cannabinoids alters male reproductive function in mice. Science 205: 1420–1422, 1979.
55. Gordon, J. H., Bromley, B. L., Gorski, R. A. and Zimmermann, E.: Delta9-tetrahydrocannabinol enhancement of lordosis behavior in estrogen treated female rats. Pharmacol. Biochem. Behav. 8: 603–608, 1978.
56. Kostellow, A. B., Ziegler, D., Kunar, J., Fujimoto, G. I. and Morrill, G. A.: Effect of cannabinoids on estrous cycle, ovulation, and reproductive capacity of female A/J mice. Pharmacology 21: 68–75, 1980.
57. Kumar, R., Mumford, L. and Teixeira, A. R.: Sexual behavior in morphine-dependent rats [proceedings]. Br. J. Pharmacol. 62: 389P–390P, 1978.
58. Mumford, L. and Kumar, R.: Sexual behaviour of morphine-dependent and abstinent male rats. Psychopharmacologie (Berlin) 65: 179–185, 1979.
59. Ostrowski, N. L., Stapleton, J. M., Noble, R. G. and Reid, L. D.: Morphine and naloxone's effects on sexual behavior of the female golden hamster. Pharmacol. Biochem. Behav. 11: 673–681, 1979.
60. Rosenkrantz, H.: Effects on cannabis on fetal development of rodents. Adv. Biosci. 22–23: 479–499, 1978.
61. Vorhees, C. V., Brunner, R. L. and Butcher, R. E.: Psychotropic drugs as behavioral teratogens. Science 205: 1220–1225, 1979.
62. Olusi, S. O., Oke, O. L. and Odusote, A.: Effects of cyanogenic agents on reproduction and neonatal development in rats. Biol. Neonate 36: 233–243, 1979.
63. Palmer, A. K., Cozens, D. D., Spicer, E. J. and Worden, A. N.: Effects of lindane upon reproductive function in a three-generation study in rats. Toxicology 10: 45–54, 1978.

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